Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 604–608

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Application of automated serial blood sampling and dried blood spot technique with liquid chromatography–tandem mass spectrometry for pharmacokinetic studies in mice

Philip Wong*, Roger Pham, Carl Whitely, Marcus Soto, Kevin Salyers, Christopher James, Bernd A. Bruenner

Department of Pharmacokinetics and Drug Metabolism, Amgen, Thousand Oaks, CA 91320-1799, United States

ARTICLE INFO

Article history:
Received 15 February 2011
Received in revised form 24 June 2011
Accepted 27 June 2011
Available online 1 July 2011

Keywords:
Dried blood spots
Automated blood sampling
LC–MS/MS

ABSTRACT

The goal of this work was to obtain full pharmacokinetic profiles from individual mice with the use of an automated blood sampling system and dried blood spot (DBS) technique. AMG 517, a potent and selective vanilloid receptor (VR1) antagonist, was dosed to mice ($n = 3$) intravenously and blood samples were collected using the automated blood sampling system with the "no blood waste" method. The collected blood samples were a mixture of 25 μL blood and 50 μL of heparinized saline solution. Two 15 μL aliquots were manually spotted onto a DBS card and dried at room temperature for at least 2 h before being stored in zip bags with desiccant. The remaining samples (45 μL) were stored at −70 °C until analysis. Both the DBS and the whole blood samples (diluted with saline (1:2, v/v)) were extracted and analyzed by liquid chromatography–tandem mass spectrometry. The overall extraction recovery of the analyte from the dried blood spots was determined to be about 90%. The pharmacokinetic parameters calculated using the whole blood or the DBS concentration data were comparable, and were obtained from only 3 mice, whereas conventional sampling and analysis would have required up to 27 mice to achieve the same result. The analyte was shown to be stable in the diluted whole blood (blood:saline 1:2) at room temperature for at least 4 h and in the DBS for at least 34 days when stored at room temperature. These results indicated that the automated blood sampling system and DBS collection are promising techniques to obtain full pharmacokinetic profiles from individual mice and reduce the use of animals.

1. Introduction

Pharmacokinetic (PK) studies in mice can require sacrifice of one animal for each time point in order to collect enough plasma for analysis, thus resulting in use of a large number of animals. Additional variability in the PK data obtained can also result from such study designs. An alternative approach is serial blood sampling in mice, which has been reported [1,2] using manual blood collection via either tail or saphenous veins. Such manual processes can introduce stress to the animals and affect pharmacokinetic results by reducing absorption or altering metabolism [3]. Automated blood sampling systems such as the Culex® system [4] were designed to facilitate pharmacokinetic and toxicokinetic studies by automating the collection of blood from conscious and freely moving animals. Automation also provides the ability to collect blood samples at time points when staff scheduling would be problematic.

Plasma has commonly been used for determining drug concentration in pharmacokinetic studies. This matrix requires a relatively large volume (100–500 μL) of whole blood to obtain enough plasma for analysis. The dried blood spot (DBS) technique [5–7] offers the potential to use low blood sample volumes (15–25 μL) making it particularly attractive for use in mouse studies where blood volume is very limited, and supporting the overall objectives of the 3Rs (reduce, refine and replace) [8] to decrease animal use. Reported use of DBS sampling dates back to 1963 when it was first used for the screening of neonatal metabolic disorders [9]. DBS has recently received considerable attention for use in the context of therapeutic drug monitoring and pharmacokinetic studies [10–17]. In addition to reduced sample volumes, DBS also offers additional benefits including reduced biohazard risk and lowering costs by simplifying sample storage and transport procedures. One potential issue with DBS is that it effectively changes the matrix used for bioanalysis from the plasma to whole blood. The pharmacokinetic implications of using DBS have been discussed in two recent publications [18,19], concluding that DBS could be effectively used for many compounds. In addition, regulatory guidance indicates that whole blood is an acceptable biological matrix for the

* Corresponding author. Tel.: +1 805 313 4701; fax: +1 805 499 4868.
E-mail address: pwong@amgen.com (P. Wong).

0731-7085/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.jpba.2011.06.022

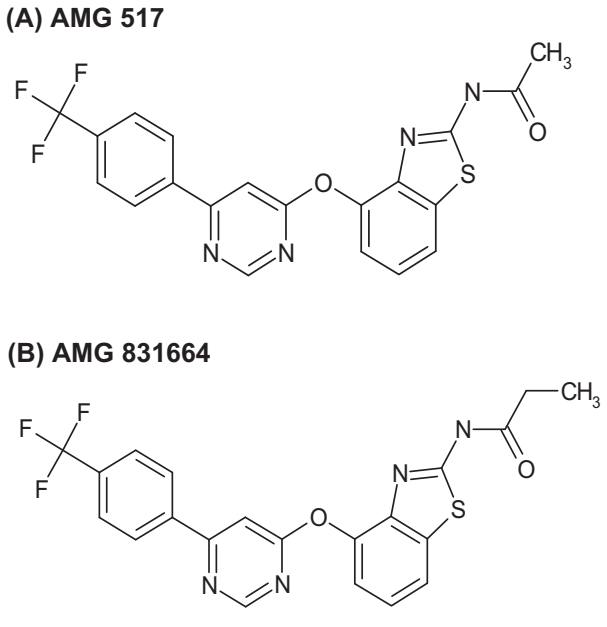

Fig. 1. Structures of (A) AMG 517 and (B) AMG 831664.

measurement of drug exposure in regulated preclinical and clinical studies [20,21].

In this study, we evaluated the use of the Culex® system and DBS technique with LC–MS/MS in obtaining full pharmacokinetic profiles from individual mice using AMG 517 (Fig. 1a) [22] as a model compound. The pharmacokinetic parameters obtained from DBS were compared with data from diluted whole blood samples (diluted with saline (1:2, v/v)). The animal sampling and bioanalytical approaches were targeted to rapidly generate data for discovery research, and consequently methods were not validated.

2. Experimental

2.1. Chemicals and materials

AMG 517 and AMG 831664 (Fig. 1b), an analog internal standard (IS), were synthesized at Amgen (Thousand Oaks, CA). Methanol, acetonitrile, dimethyl sulfoxide (DMSO), formic acid and ammonium formate were obtained from Sigma–Aldrich. Saline was supplied by Hospira Inc. (Lake Forest, IL) and the sodium heparin (10,000 U/mL) by APP Pharmaceuticals (Schaumburg, IL). The sodium heparin was diluted with saline to provide a heparin–saline solution of 20 U/mL for the study. Fresh mouse whole blood (sodium heparin anticoagulant) was collected on the day of use and was supplied by the Department of Pharmacokinetics and Drug Metabolism at Amgen. DBS cards (FTA DMPK A card), punch mats and 6-mm punch tools were obtained from GE Healthcare (Piscataway, NJ).

2.2. Calibration standards

Stock solutions of AMG 517 and AMG 831664 (1.0 mg/mL) were prepared in DMSO. Dilution of the stock solutions (1.0 mg/mL) with methanol/water (1:1) yielded working solutions of 100 μg/mL. A 30 μL aliquot of 100 μg/mL AMG 517 was spiked into 570 μL of mouse whole blood to obtain a 5000 ng/mL calibration standard in whole blood. Serial dilutions of the 5000 ng/mL calibration standards were prepared to provide standards in the range of 0.5–2000 ng/mL. The calibration standards (0.5, 1, 2.5, 5, 25, 100, 250, 500, 1000, 2000 and 5000 ng/mL) in whole blood were then mixed with heparinized saline solution in a ratio of 1:2 (v/v) to mimic the collected blood samples from the Culex® system (Bioanalytical Systems, West Lafayette, IN) and were run in duplicate in each analytical batch. For DBS analyses, 15 μL of the diluted blood/saline (1:2) samples were spotted on DBS cards using a pipette. The pipette tip was kept from touching the spotting area when applying the samples. The samples were allowed to dry at room temperature for at least 2 h on a drying rack. Concentration data in all experiments were reported in terms of undiluted whole blood concentration. A working internal standard solution of AMG 831664 (20 ng/mL) was prepared in 75% methanol/water (v/v).

2.3. 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. A total of 3 male mice (CD-1, 6–8 weeks old), surgically implanted with a carotid artery catheter, were supplied by Taconic (Albany, NY). Mice were dosed intravenously at 1.0 mg/kg of AMG 517 in DMSO. Blood samples were collected at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 24 h post-dose by the Culex® system using the "no blood waste" method in which heparinized saline (50 μL) was used to deliver the collected whole blood (25 μL) into a vial stored in a refrigerated fraction collector (4 °C). The resulting samples were a mixture of 25 μL blood and 50 μL heparinized saline. The Culex® system was calibrated by the manufacturer with accuracy within 95% or better and a precision of ±0.4 μL for a volume of 20 μL or greater. Two 15 μL aliquots of the collected samples were manually spotted on DBS cards and allowed to dry at room temperature for at least 2 h before being stored in zip bags with desiccant. The remaining samples (45 μL) were stored at −70 °C until analysis.

2.4. Sample preparation

For DBS analyses, a 6-mm diameter disk was punched from the center of the DBS card into a 1.4-mL cluster tube (part # MP226RN, Micronic BV, McMurray, PA). A 100 μL aliquot of the working internal standard was added and the tube was vortex mixed for about 30 min. The tube was centrifuged at 4000 rpm (∼3000 × *g*-force) for 10 min and the supernatant was transferred into a 96-well plate. The supernatant (10 μL) was then injected into the LC–MS/MS system.

For blood/saline analyses, 100 μL of the working internal standard was added into a 15 μL aliquot of the blood/saline (1:2) samples in a 1.4-mL cluster tube. The tube was then vortex mixed for 1 min and centrifuged for 10 min at 4000 rpm. The supernatant was transferred into a 96-well plate and 10 μL of the supernatant was injected into the LC–MS/MS system.

2.5. Analytical instrumentation

All experiments were performed using an API 5000 triple quadrupole mass spectrometer (AB Sciex, Foster city, CA) controlled by Analyst 1.4.1 software. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with positive ion atmospheric pressure chemical ionization. The MRM transitions were 431.1 → 389.1 and 445.1 → 214.0 with a dwell time of 100 ms for AMG 517 and AMG 831664 respectively. The instrument was directly coupled to a Shimadzu Prominence HPLC system (Shimadzu, Japan) including a Shimadzu SIL-20AD UFLCXR solvent delivery system and a SIL-20AC XR autosampler. Results from the API 5000 were imported into the Watson LIMS system (version 7.0, Thermo Scientific, Philadelphia, PA) for data processing. Calibra-

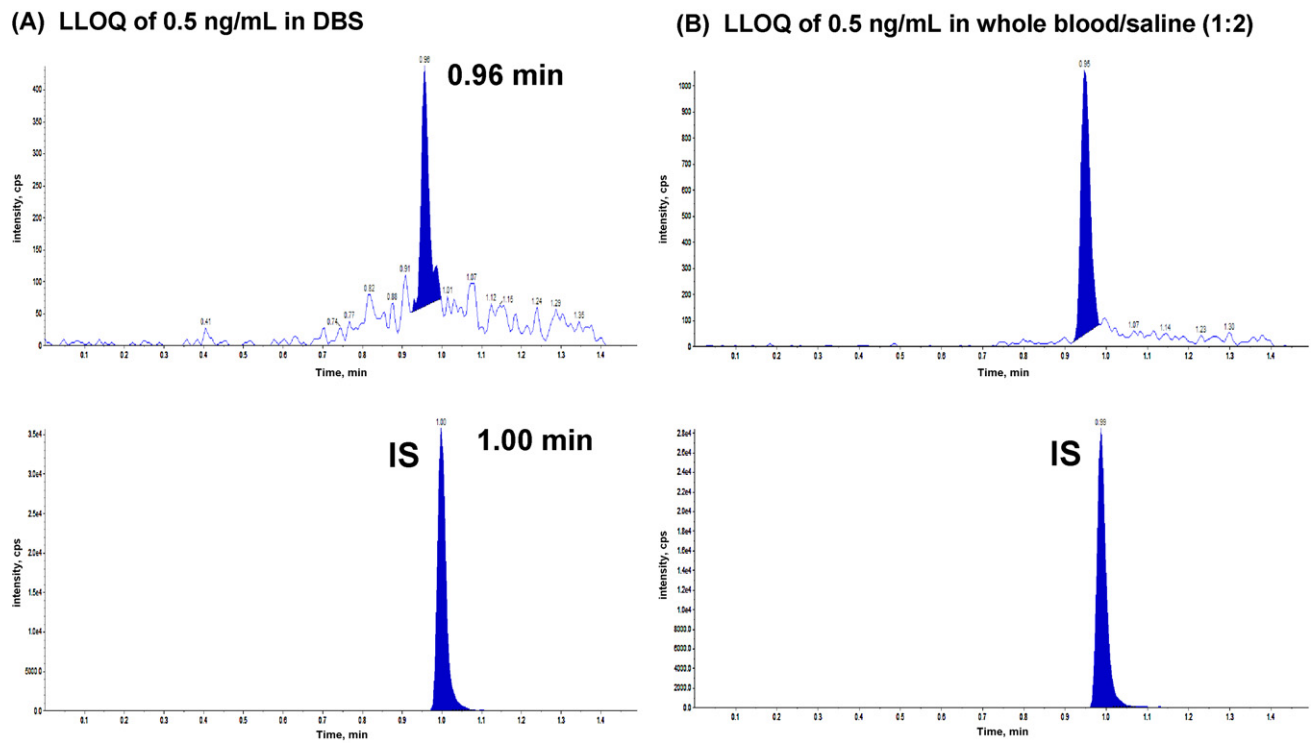

Fig. 2. Chromatograms of the lower limit of quantitation (LLOQ, 0.5 ng/mL) of AMG 517 in (A) DBS and (B) diluted blood samples.

tion curves were constructed with a weighted 1/concentration^2 quadratic regression.

2.6. 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 Waters XTerra MS C_{18} (50 mm × 2.1 mm, 2.5 μm) analytical column which was 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 0.2–0.65 min, and was held constant until 1.1 min. At 1.11 min, the gradient was changed back to the starting conditions. The total run time was about 1.5 min.

2.7. Recovery experiments

Extraction recovery from the DBS was evaluated at 5 and 1000 ng/mL in whole blood with triplicate analysis. Additional blank blood/saline (1:2) samples were extracted to provide control extracts for use in recovery experiments. Aliquots (6 μL) of blood/saline samples at blood concentrations of 5 and 1000 ng/mL were spotted onto DBS cards and dried at room temperature for at least 2 h. The blood spots formed by the 6 μL samples have 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 in Section 2.4. 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 6 μL spot at 5 and 1000 ng/mL concentration. Recovery was calculated by comparing the response of the extracted samples with the spiked post-extracted blank samples.

2.8. Stability in diluted blood samples

The stability of AMG 517 in diluted blood samples (blood:saline 1:2) was evaluated by storing diluted blood samples at concentrations of 5 and 1000 ng/mL for 4 h at room temperature. The samples (n = 6 at each concentration) were analyzed as described in Section 2.4 and the results were compared with those of fresh samples.

2.9. Stability on DBS cards

The stability of AMG 517 on DBS cards was evaluated by storing the DBS samples at concentration of 5 and 1000 ng/mL for 34 days at room temperature in a zip bags with a desiccant. The samples (n = 6 at each concentration) were analyzed as described in Section 2.4 and the results were compared with those of fresh samples.

3. Results and discussion

3.1. DBS and whole blood concentration data comparison

Fig. 2 shows chromatograms at the lower limit of quantitation (LLOQ, 0.5 ng/mL) of AMG 517 in the DBS and the diluted blood samples. A 6-mm diameter punch of the DBS is equivalent to a volume of about 7.7 μL of the diluted blood sample. This is about half of the volume used in the analysis of the diluted whole blood samples (15 μL). This explains why the response in the DBS samples is about half of the whole blood samples. However, the sensitivity at the LLOQ for the DBS samples was still sufficient for quantitation. The correlation coefficients (r^2) for the calibration curves of the diluted whole blood and DBS are 0.9920 and 0.9934 respectively with accuracy within ±20%. Table 1 shows the concentration–time data for both the DBS and the whole blood after an IV dosing of 1.0 mg/kg of AMG 517 to mice. The average difference between the two sets of concentration data was about 12%; Fig. 3 shows the

Table 1
Concentration of AMG 517 in DBS and whole blood.

Time (h)	Concentration in DBS (ng/mL)			Concentration in whole blood (ng/mL)			% Difference in subject 1	% Difference in subject 2	% Difference in subject 3
	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3			
0.083	2090	1330	637	1880	1600	623	10.6	−18.4	2.2
0.25	1530	903	585	1850	863	740	−18.9	4.5	−23.4
0.5	1030	621	493	1290	731	598	−22.4	−16.3	−19.2
1	885	513	347	897	539	426	−1.3	−4.9	−20.4
2	727	382	291	717	379	342	1.4	0.8	−16.1
4	691	N/A[a]	132	829	N/A[a]	126	−18.2	N/A	4.7
6	607	98.3	106	770	88.1	133	−23.7	10.9	−22.6
8	601	60.9	98.9	646	49.7	95.7	−7.2	20.3	3.3
24	357	37.0	73.5	400	36.2	78.0	−11.4	2.2	−5.9

[a] No sample was collected.

Table 2
Average (±SD) pharmacokinetic parameters generated from both the DBS and whole blood concentration data ($n = 3$).

Matrix	$AUC_{(0-24h)}$ (ng*h/mL)	Clearance (mL/kg/h)	Volume of distribution (mL/kg)	Mean residence time (h)
Whole blood	7070 (±6870)	148 (±110)	3670 (±2370)	27.9 (±11.0)
DBS	6500 (±6060)	150 (±112)	3900 (±2710)	30.4 (±15.5)

plots of concentration–time profiles. The pharmacokinetic parameters were calculated using non-compartmental analysis in Watson LIMS System and are tabulated in Table 2. The large standard deviations were due to the large difference in the concentration profile in one animal as shown in Fig. 3. Pharmacokinetic parameters calculated using data from both methods were similar. For example, the average AUC_{0-24h} values were 7070 and 6500 ng*h/mL obtained by using the whole blood and DBS concentration data, respectively, a difference of about 8.4%. Average clearance values were 148 and 150 mL/kg/h, and average volumes of distribution were 3670 and 3900 mL/kg, for whole blood and DBS data, respectively. The agreement between the whole blood and DBS data suggested that DBS sampling is a promising technique for reducing blood volume requirements and thus the number of animals used in pharmacokinetic studies. Using one mouse for each time point would have required 27 mice to generate similar data that was obtained with only three animals in this study.

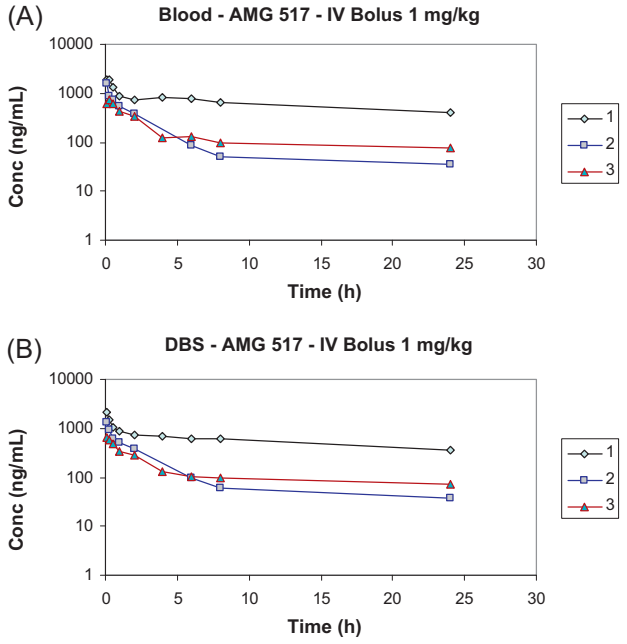

Fig. 3. Plots of concentration–time profiles generated using (A) the whole blood data and (B) the DBS data.

3.2. Recovery

The extraction recoveries of AMG 517 from DBS samples were determined at 5 and 1000 ng/mL and were found to be 94 and 87% respectively. The effect of vortex extraction time (10, 20, 30 and 60 min) was also studied and it was found that longer extraction time (60 min) did not increase the extraction recovery (Fig. 4), although greater variability was seen if the discs were only vortexed for 10 min.

3.3. Stability in diluted blood samples

Stability in diluted blood was studied to allow manipulation of blood samples prior to spotting onto a card. The stability of the diluted blood samples was evaluated at 5 and 1000 ng/mL for up to 4 h at room temperature. The LC–MS/MS peak area ratio (analyte to internal standard) of replicates ($n = 6$) of these samples stored for 4 h at room temperature were compared with those of aliquots of fresh samples. The difference in peak area ratios were −1.1% and −7.4% at 5 ng/mL and 1000 ng/mL respectively, indicating that AMG 517 is stable in diluted blood samples for at least 4 h at room temperature.

3.4. Stability on DBS cards

Replicates ($n = 6$) of 15 μL of the diluted blood samples (blood:saline 1:2) at 5 and 1000 ng/mL were spotted onto a card

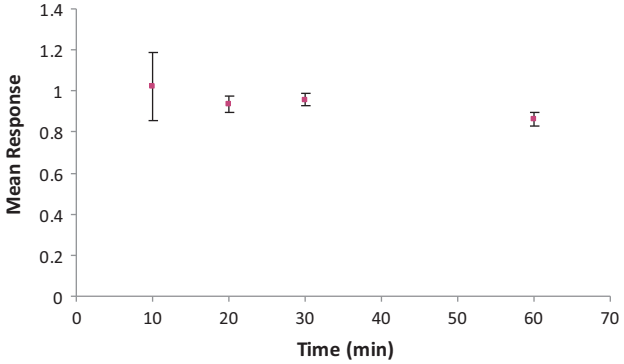

Fig. 4. Effect of vortex extraction time on DBS recovery of AMG 517 at 1000 ng/mL ($n = 3$).

and stored at room temperature for 34 days in a zip bag with a desiccant. The measured responses (peak area ratios) were compared to fresh samples extracted and analyzed immediately after initial spotting and drying. The differences were 9.4 and 12.0% at 5 and 1000 ng/mL, respectively, indicating AMG 517 is stable on DBS cards for at least 34 days when stored at room temperature with a desiccant.

4. Conclusion

This report describes the application of an automated serial blood sampling system used in conjunction with DBS collection for pharmacokinetic studies in mice. Calculated parameters such as AUC and clearance determined from DBS and diluted whole blood methods were comparable. The high extraction recovery and adequate stability on DBS cards also suggest that DBS is a promising technique in reducing the blood volume requirements for pharmacokinetic studies. These results also demonstrated that the automated blood sampling system and DBS collection can provide full pharmacokinetic profiles from individual mice and reduce the number of animals required for these types of studies.

Acknowledgements

The authors would like to thank Candace Rohde and Scott Peters from Bioanalytical Systems (West Lafayette, IN) for technical support of the Culex® automated blood sampling system.

References

[1] K.P. Bateman, G. Castonguay, L. Xu, S. Rowland, D.A. Nicoll-Griffith, N. Kelly, C.C. Chan, Reduction of animal usage by serial bleeding of mice for pharmacokinetic studies: application of robotic sample preparation and fast liquid chromatography–mass spectrometry, J. Chromatogr. B. 754 (2001) 245–251.
[2] S.X. Peng, B.A. Rockafellow, T.M. Skedzielewski, N.D. Huebert, W. Hageman, Improved pharmacokinetic and bioavailability support of drug discovery using serial blood sampling in mice, J. Pharm. Sci. 98 (2009) 1877–1884.
[3] F. Jamali, C.M. Kunz-Dober, Pain-mediated altered absorption and metabolism of ibuprofen: an explanation for decreased serum enantiomer concentration after dental surgery, Br. J. Clin. Pharmacol. 47 (1999) 391–396.
[4] P.C. Gunaratna, P.T. Kissinger, C.B. Kissinger, J.F. Gitzen, An automated blood sampler for simultaneous sampling of systemic blood and brain microdialysates for drug absorption, distribution, metabolism, and elimination studies, J. Pharmacol. Toxicol. 49 (2004) 57–64.
[5] W. Li, F.L.S. Tse, Dried blood spot sampling in combination with LC–MS/MS for quantitative analysis of small molecules, Biomed. Chromatogr. 24 (2010) 49–65.
[6] P. Patel, H. Mulla, S. Tanna, H. Pandya, Facilitating pharmacokinetic studies in children: a new use of dried blood spots, Arch. Dis. Child. 95 (2010) 484–487.
[7] P. Wong, R. Pham, B.A. Bruenner, C. James, Increasing efficiency for dried blood spot analysis: prospects for automation and simplified sample analysis, Bioanalysis 2 (2010) 1787–1789.
[8] W.M.S. Russel, L.R. Burch, Principle of Human Experimental Technique, Methuen, London, 1959.
[9] R. Guthrie, A. Susi, A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants, Pediatrics 32 (1963) 338–343.
[10] N. Spooner, R. Lad, M. Barfield, Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method, Anal. Chem. 81 (2009) 1557–1563.
[11] X. Liang, Y. Li, M. Barfield, Q. Ji, Study of dried blood spots technique for the determination of dextromethorphan and its metabolite dextrorphan in human whole blood by LC–MS/MS, J. Chromatogr. B 877 (2009) 799–806.
[12] M. Barfield, Neil Spooner, R. Lad, S. Parry, S. Fowles, Application of dried blood spots combined with LC–MS/MS for the quantification of acetaminophen in toxicokinetic studies, J. Chromatogr. B. 870 (2008) 32–37.
[13] P.M. Edelbroek, J.V. Heijden, L.M. Leo, Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls, Ther. Drug Monit. 31 (2009) 327–336.
[14] G.T Clark, J.J. Haynes, M.A.J. Bayliss, L. Burrows, Utilization of DBS within drug discovery: development of a serial microsampling pharmacokinetic study in mice, Bioanalysis 2 (2010) 1477–1488.
[15] N. Youhnovski, J. Michon, S. Latour, J. Mess, A. Bergeron, M. Furtado, M. Rufiange, P. Guibord, M. Lefebvre, R.B. MacArthur, F. Garofolo, Determination of naproxen using DBS: evaluation & pharmacokinetic comparison of human plasma versus human blood DBS, Bioanalysis 2 (2010) 1501–1513.
[16] N. Spooner, Y. Ramakrishnan, M. Barfield, O. Dewit, S. Miller, Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations, Bioanalysis 2 (2010) 1515–1522.
[17] P.V. Amsterdam, C. Waldrop, The application of dried blood spot sampling in global clinical trials, Bioanalysis 2 (2010) 1783–1786.
[18] M. Rowland, G.T. Emmons, Use of dried blood spots in drug development: pharmacokinetic considerations, AAPS J. 12 (2010) 290–293.
[19] G. Emmons, M. Rowland, Pharmacokinetic considerations as to when to use dried blood spot sampling, Bioanalysis 2 (2010) 1791–1796.
[20] Note for Guidance on Toxicokinetics: The assessment of systemic exposure in toxicity studies, ICH Harmonised Tripartite Guideline, Code S3A, 1994.
[21] Toxicokinetics: Guidance on the Assessment of Systemic Exposure in Toxicity Studies, FDA Fed. Regis. 60 (1995) 11264–11268.
[22] A. Bak, A. Gore, E. Yanez, M. Stanton, S. Tufekcic, R. Syed, A. Akrami, M. Rose, S. Surapaneni, T. Bostick, A. King, S. Neervannan, D. Ostovic, A. Koparkar, The co-crystal approach to improve the exposure of a water-insoluble compound: AMG 517 sorbic acid co-crystal characterization and pharmacokinetics, J. Pharm. Sci. 97 (2008) 3942–3956.