Journal of Chromatography B, 879 (2011) 1713–1716

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

Dried blood spot assay for estimation of metronidazole concentrations in rats and its application in single animal drug pharmacokinetic study

Prashant Laxman Kole[a,*], Rita Majithia[b], Thakur Raghu Raj Singh[b], Martin J. Garland[b], Katarzyna Migalska[b], Ryan F. Donnelly[b], James McElnay[a]

[a] PKPD Core Facility, School of Pharmacy, Medical Biology Centre, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, United Kingdom
[b] Drug Delivery and Biomaterials Group, School of Pharmacy, Medical Biology Centre, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, United Kingdom

ARTICLE INFO

Article history:
Received 21 January 2011
Accepted 31 March 2011
Available online 8 April 2011

Keywords:
Dried blood spot
Single animal pharmacokinetic study
Ahlstrom 226
Metronidazole

ABSTRACT

An HPLC-UV-dried blood spot (DBS) method for the estimation of metronidazole (MTZ) in rat whole blood is reported. Method employs Ahlstrom 226 sample collection paper and DBS samples were prepared by spotting with 30 μl of whole blood (spiked calibration standards/quality control samples/in vivo study samples). A 6 mm disc was punched from each DBS and extraction was carried out using water containing the internal standard (tinidazole). The calibration for MTZ was linear over 2.5–50 μg/ml concentration range. Accuracy (% bias) and precision (expressed as % Coefficient of variation) values for within and between day were <20% at the lower level quality control sample (LQC) and <15% at all other concentrations tested. The limit of quantification (LOQ) of the method was 2.5 μg/ml. The validated method was applied for the analysis of in vivo pharmacokinetic (PK) study samples after intravenous administration of MTZ to a rat. Whole blood PK parameters observed in this study were in compliance with literature based PK parameters. The DBS sampling approach was found to be useful in a single animal pharmacokinetic study.

1. Introduction

Traditional systematic pre-clinical pharmacokinetic (PK) studies employing rodents, such as mouse/rats, involve parallel blood sampling i.e. collection of one/two blood samples of 0.5–1 ml volume from single animal. This volume is required to obtain sufficient plasma/serum for bioanalysis. However, limitation of parallel sampling is the fact that the number of animals required can be large depending upon the numbers of time points and/or replicates so as to obtain reliable and accurate PK data. One technical alternative to reduce the number of animals and variability in PK data is to adopt serial blood sampling, wherein a number of blood samples are taken from a single animal. However, traditional bioanalytical methods require large volumes of biomatrix to accurately estimate the drug/metabolite concentrations. Withdrawal of large volumes of blood at each time point leads to haemodynamic changes in animal physiology, thus limits the number of samples that can be taken from a single animal [1].

In the recent past, emphasis has been given to the 3Rs policy in animal use i.e. Reduce, Refine and Replace animal use in research [2]. Compliance with this 3Rs policy poses important challenges to PK study design and bioanalysis. The collection of very low volume blood samples (10–30 μl) on absorbent paper, known as the dried blood spot (DBS) technique, is one appropriate strategy to achieve these objectives. DBS offers several advantages when compared to conventional venipuncture, such as being relatively non-invasive, simple to perform, requires minimal training, enables room temperature transport/storage since most analytes are stabilised in a dry matrix, easy transportation and flexibility in collection of blood samples off-site [3]. Use of DBS in preclinical PK studies improves PK data quality (as it enables serial samples from a single animal), significantly reduces number of animals required and offer significant ethical and cost benefits [4]. These benefits of DBS are possible because of very low blood volume sampled at each time point.

The bioanalytical method presented here is a part of PK evaluation of our novel microneedle-based drug delivery system [5,6] employing metronidazole (MTZ) as a model drug. Before microneedle administration studies, it was essential to establish basic PK parameters of the MTZ in the proposed animal model i.e. rats. Based on the objectives of the project, the aim of the proposed DBS method was to establish a simple and robust bioanalytical method employing the lowest possible sampling volume, ease of sample preparation and adequate selectivity and sensitivity. Furthermore, the second most important objective was feasibility assessment of

* Corresponding author. Tel.: +44 28 90972361; fax: +44 28 90247794.
E-mail addresses: prashantkole@gmail.com, p.kole@qub.ac.uk (P.L. Kole).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.03.060

use of whole blood (in the form of DBS) in determination of PK parameters of MTZ in comparison with a conventional biomatrix i.e. plasma/serum. To assist with the development of the study protocol, a detailed literature search was carried out for use of the DBS approach in animal PK studies and various bioanalytical methods reported for estimation of MTZ in rat whole blood/plasma. This review of the literature indicated very few reports [4,7,8], which employed the DBS technique in pre-clinical studies and illustrated the need for further investigations [9]. Literature on bioanalytical methods for quantification of MTZ in the rat indicated only three HPLC methods employing UV detection [10–12].

We earlier reported a DBS method for estimation of MTZ in neonatal and paediatric blood samples using Guthrie cards (Schleicher & Schuell 903®) [13]. Methodology from our previous work was further investigated for estimation of MTZ in rat DBS samples. However, instead of Schleicher & Schuell 903® sampling paper, Ahlstrom 226 specimen collection paper was employed. Two study parameters i.e. change of bio matrix (human blood versus rat blood) and type of sampling paper (Ahlstrom 226 instead of Schleicher & Schuell 903®) were varied as compared to our previously reported method [13]. The DBS method reported here was re-validated before being applied to an in vivo study of MTZ in rats.

2. Materials and methods

2.1. Chemicals and materials

MTZ and tinidazole were purchased from Sigma–Aldrich (Poole, England). HPLC grade methanol, acetonitrile, Potassium dihydrogen phosphate were purchased from BDH Chemicals Ltd. (Poole, England). HPLC grade water was produced using a Millipore Direct-Q™ water purification system (Millipore, Watford, England). Ahlstrom 226 sample collection papers were purchased from ID biological systems, UK. The punch used to cut out the DBS was a Sole 6 mm punch [Model PF35A0G1, Rapesco Sevenoaks, England). Drug free whole blood for method development and validation was collected from healthy Sprague Dawley rats. Protocol for the collection of blank blood and the in vivo study was approved by the Ethical Review Committee for Animal research, Queen's University Belfast, UK.

2.2. Preparation of stock solutions, calibration standards, quality control (QC) samples

A primary stock solution (PS) of MTZ (10 mg/ml) was prepared by dissolving 100 mg in 10 ml of methanol. Working standard solutions (WS) of MTZ at concentrations 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2 and 2.5 mg/ml were prepared by appropriate dilution of PS with methanol. PS and WS were stored in refrigerator (4 °C) until used. Calibration standards (CS) of MTZ were prepared by spiking 20 μl of WS in 980 μl of blank whole rat blood to yield 2.5, 5, 10, 15, 20, 30, 35, 40 and 50 μg/ml concentrations. Tinidazole (TNZ) was used as an internal standard (IS). When preparing the samples for extraction, 25 μl of this diluted stock (8 μg/ml) was added to the extraction mixture (0.975 ml water) to produce an internal standard concentration of 0.2 μg/ml. For validation purpose, three QC concentrations *viz*. low (LQC, 2.5 μg/ml), medium (MQC, 20 μg/ml) and high (HQC, 50 μg/ml) were selected and were prepared using the same procedure as that of CS samples. Analytical standards (AS) at similar concentration as that of CS were also prepared in water for determination of the absolute recovery of MTZ.

2.3. Blood spotting and sample preparation

The DBSs were prepared by spotting 30 μl of the respective spiked CS/QC or whole blood from the MTZ treated rats onto sampling paper using a calibrated pipette. The samples were left to dry in the dark for at least 3 h before storing at controlled room temperature (CRT, 20 ± 2 °C) until analysis. CS and QC samples thus prepared were used in the validation of the method.

For sample preparation, a 6 mm disc was punched from the DBS, ensuring that an area completely filled with blood was obtained. The disc was transferred to an eppendorf tube and mixed with 975 μl of water and 25 μl of the IS. The sample was vortex-mixed for 10 s at 10-min intervals for 30 min period. After the extraction period, samples were centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to an auto-sampler vial for analysis.

2.4. Chromatographic system and conditions

The chromatography was carried out using the Agilent HPLC system (1200 series, Cheshire, UK), consisting G1312B Binary Pump and 1200 VWD UV-visible detector. Chemstation software enabled the control of operating parameters, data capture, process and storage. The separation was performed using a Waters Symmetry® C18 column (5 μm, 3.9 mm × 150 mm) preceded by guard column (5 μm, 3.9 mm × 20 mm) of matching chemistry. The mobile phase consisted of acetonitrile/0.01 M potassium dihydrogen phosphate buffer (pH 4.7) in 15:85, v/v ratio, pumped at a flow rate of 1 ml/min. The injection volume was 80 μl and UV detection was carried out at 317 nm. The total analysis time per sample was 7.5 min.

2.5. Method validation

The developed DBS method was validated according to ICH guidelines [14]. Selectivity of the method was assessed by process-

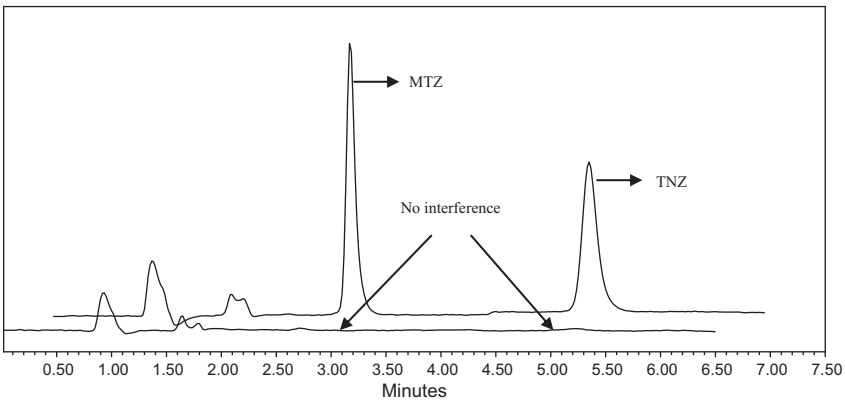

Fig. 1. Overlay chromatogram of blank and CS (20 μg/ml) DBS sample.

Table 1
Accuracy and precision data of MTZ DBS method ($n = 3$).

Concentration (μg/ml)	Acceptance limit (%)	Intra-day accuracy (% bias)	Inter-day accuracy (% bias)	Intra-day precision (% CV)	Inter-day precision (%CV)
LQC (2.5)	<±20	7.21	5.31	6.52	4.65
MQC (20)	<±15	5.34	−3.63	6.17	5.51
HQC (50)	<±15	−5.68	−4.46	5.29	6.03

ing and analysing blank DBS samples from six independent sources. The linearity of the developed method was evaluated by preparing five calibration curves for the analyte over five consecutive days. Accuracy (expressed as % bias) and precision (expressed as % CV) of the three spiked QC samples i.e. LQC, MQC and HQC were assessed over five days ($n = 3$). The efficiency of the extraction procedure was determined by the analysis of DBS spiked with the analyte at three QC concentrations (2.5, 20 and 50 μg/ml). Five replicates at each concentration level were extracted and analysed and the responses compared with those of non-extracted standards, which represent 100% recovery. Stability of MTZ and TNZ stock solutions stored at refrigerated condition (4 °C) was assessed for a period of one month. Controlled room temperature stability of MTZ QC samples at three levels i.e. LQC, MQC and HQC ($n = 3$) was assessed for a period of one month. Further, post-preparation bench top stability of the processed samples of MTZ at three QC concentrations (LQC, MQC and HQC) was assessed for up to 8 h.

2.6. Application of DBS method for in vivo PK study

The validated DBS method was applied for in vivo PK study samples. The study involved intravenous administration (10 mg/kg) of MTZ to three overnight fasted male Sprague Dawley rats (average weight 275 ± 20 g). At pre-determined time intervals (0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 32, 48 h post administration), 75 μl of blood sample was taken from the tail vein and transferred to EDTA coated tubes (Microvette CB300, 300 μl EDTA, Sarstedt, USA). After gentle mixing with anti-coagulant, two DBS were prepared from each time point. The samples were processed, as per the procedure detailed in Section 2.3 and analysed. The concentration of MTZ versus time data thus obtained was further subjected to compartmental modelling (WinNonlin version 2.1, Pharsight, USA) to determine various PK parameters. The PK data obtained from DBS was further compared with previously reported PK data in rats.

3. Results and discussion

3.1. Method development

As mentioned previously, chromatographic conditions of our previously reported HPLC method [13] were used for the current DBS method. No significant difference in the chromatographic profile of MTZ was observed when the method was transferred from Waters HPLC system to Agilent 1200 series HPLC system. Aqueous sample preparation protocol was found to be efficient for extraction MTZ from DBS. Fig. 1 shows an overlay chromatogram of blank and CS DBS sample (20 μg/ml). The retention times of MTZ and TNZ were found to be 2.9 ± 0.2 and 4.9 ± 0.2 min, respectively. The absolute recovery study indicated 58.47 ± 4.55, 59.19 ± 2.35 and 60.13 ± 3.34% recovery of MTZ at LQC, MQC and HQC respectively, as compared to previously reported average recovery of 78 ± 2% [13]. The lower recovery of MTZ in the current method as compared to our previous report was most likely due to the change in sampling paper. Differences in whole blood viscosity (WBV) may also have played a role. WBV in rats, at a normalised hematocrit value of 40%, at a shear rate of 0.7/s is reported to be 35.4 mPa s, while in humans it is 31.48 mPa s [15]. This apparent difference in blood viscosity may lead to higher matrix density around the analyte molecules in DBS, leading to reduced recovery. However, the recovery at all three QC levels was found to be consistent.

3.2. Method validation

Analysis of six independent blank DBS samples did not show any interfering peaks at the retention time of the MTZ or TNZ, indicating selectivity of the method (Fig. 1). The calibration curve (peak area response versus concentration (μg/ml)) of MTZ was found to be linear over the selected calibration range. The calibration data was subjected to least square regression analysis and the mean linear regression equation obtained for the proposed method was $Y = 0.061X + 0.066$. The correlation coefficient value was highly significant ($r = 0.9939$). Data on within day and between-day accuracy and precision of the method is presented in Table 1. Overall, accuracy and precision values for within and between-day were <20% at LQC and <15% at all other concentrations. Limit of quantification (LOQ) for the validated method was 2.5 μg/ml as the accuracy and precision values were <20%. Stock solution stability of MTZ and TNZ showed no change in assay value over a period of one month, indicating that both the drugs were stable at 4 °C. Room temperature stability study of spiked DBS samples at all three QC concentrations showed values comparable with freshly prepared DBS samples. Bench top stability of processed samples at all three QC levels showed <1% variation when responses of MTZ were compared at 0 time and at 8 h.

3.3. In vivo sample analysis and PK

The validated DBS method was successfully applied for analysis in vivo DBS study samples. Although samples were collected up to 48 h, the concentration of MTZ at 48 h was less than the LOQ and thus was not considered in the PK profiling. DBS concentration data was subjected to compartmental modelling and data fitted best to a two-compartment open model, with first-order elimination from the central compartment. The results of the current study are in agreement with earlier reports for MTZ using a conventional biomatrix i.e. plasma [12]. The mean maximum concentration (C_{max}) of MTZ was found to be 21.37 ± 3.69 μg/ml. The mean elimination rate constant (beta) was found to be 0.0436 ± 0.0071 h^{-1} and the blood half-life of MTZ during the beta-phase was found to be 16.21 ± 2.93 h. Literature on MTZ pharmacokinetics indicated plasma half-lives of 10.9 ± 1.6 (intravenous administration) and 13.6 ± 4.2 h (intravaginal administration) in rats [16]. Considering the standard deviation of the mean values, the whole blood half-life of the MTZ was in good agreement with the reference values from the literature. As the PK parameters obtained through DBS approach were in agreement with literature based values, the methodology was adopted for in vivo evaluation of microneedle drug delivery systems containing MTZ in rats.

4. Conclusions

Our previously reported and fully-validated DBS method for estimation of MTZ was re-customised and validated for its estimation in rat whole blood samples using Ahlstrom 226 sampling paper. Method transfer from different HPLC systems resulted in reproducible results. The method validation results were highly

satisfactory and complied with regulatory requirements (ICH). The method was successfully applied for a single animal in vivo PK study of MTZ in rats. Importantly, PK data based on DBS was in conformity with literature based PK data, indicating suitability of the DBS model and methodological compliance with the 3R policy. The validated DBS method is currently been used for in vivo PK studies of transdermal delivery of MTZ from MTZ-loaded soluble microneedle formulations in rats.

References

[1] G.M. Walsh, R.A. Ferrone, M. Tsuchiya, E.F. Woods, E.C. Deland, Am. J. Physiol. 239 (1980) H805.
[2] C.F. Hendriksen, ILAR J. 43 (Suppl. S43–48) (2002).
[3] M.F. Suyagh, P.L. Kole, J. Millership, P. Collier, H. Halliday, J.C. McElnay, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 878 (9–10) (2010) 769.
[4] M. Barfield, N. Spooner, R. Lad, S. Parry, S. Fowles, Chromatogr. B Analyt. Technol. Biomed. Life Sci. 870 (2008) 32.
[5] R.F. Donnelly, R. Majithiya, T.R.R. Singh, D.I.J. Morrow, M.J. Garland, Y.K. Demir, K. Migalska, R. Elizabeth, D. Gillen, C.J. Scott, A.D. Woolfson, Pharm. Res. 28 (2010) 41.
[6] P. Mikolajewska, R.F. Donnelly, M.J. Garland, D.I.J. Morrow, T.R.R. Singh, V. Iani, J. Moan, A. Juzeniene, Pharm. Res. 27 (10) (2010) 2213.
[7] T. Fujimoto, R. Tawa, S. Hirose, Chem. Pharm. Bull. (Tokyo) 37 (1989) 174.
[8] A. Thomas, J. Déglon, T. Steimer, P. Mangin, Y. Daali, C. Staub, J. Sep. Sci. 33 (2010) 873.
[9] T. Mauriala, N. Chauret, R. Oballa, D.A. Nicoll-Griffith, K.P. Bateman, Rapid Commun. Mass Spectrom. 19 (2005) 1984.
[10] J.D. Wibawa, P.N. Shaw, D.A. Barrett, J. Chromatogr. B Biomed. Sci. Appl. 761 (2001) 213.
[11] Q. Chen, S. Hou, J. Zheng, Y. Bi, Y. Li, X. Yang, Z. Cai, X. Song, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 858 (2007) 199.
[12] T. Yu-Hsing, Y. Wang, V. Loyd Allen Jr., D. Donald, M.K. Albersand, Gorgin, Int. J. Pharm. 61 (1990) 119.
[13] M.F. Suyagh, G. Iheagwaram, P.L. Kole, J. Millership, P. Collier, H. Halliday, J.C. McElnay, Anal. Bioanal. Chem. 397 (2010) 687.
[14] The European Agency for the Evaluation of Medicinal Products, ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Methodology. ICH Topic Q2B (CPMP/ICH/281/95), The European Agency for the Evaluation of Medicinal Products, London, 1996.
[15] U. Windberger, A. Bartholovitsch, R. Plasenzotti, K.J. Korak, G. Heinze, Exp. Physiol. 88 (2003) 431.
[16] H.S. Buttar, W.H. Siddiqui, Arch. Int. Pharmacodyn. Ther. 245 (1980) 4.