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Development and validation of a dried blood spot LC–MS/MS assay to quantify ranitidine in paediatric samples

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ABSTRACT

A novel approach has been developed to determine ranitidine in paediatric samples using dried blood spots (DBS) on Guthrie cards (Whatman 903). A selective and sensitive HPLC–MS/MS assay has been developed and validated using small volumes of blood (30 μl). A 6 mm disc was punched from each DBS and extracted with methanolic solution of the internal standard (IS) nizatidine. This was further subjected to solid phase extraction (SPE), followed by reversed phase HPLC separation, using a XBridge™ C18 column and mobile phase 10 mM ammonium acetate/methanol (98:2 v/v) with a flow rate of 0.3 mL/min. This was combined with multiple reaction monitoring (MRM) mass detection using electrospray ionisation (ESI). The calibration curve for ranitidine was found linear over the range 10–500 ng/mL (r = 0.996). The limit of quantification (LOQ) of the method was validated at 10 ng/mL. Accuracy and precision values for within and between days were <20% at the LOQ and <15% at all other concentrations. The validated DBS method was successfully applied to a clinical study employing 81 samples from 36 paediatric patients.

1. Introduction

Ranitidine (Fig. 1a) is a histamine H_2-receptor antagonist that inhibits stomach acid production. It is commonly used in treatment of peptic ulcer disease (PUD) and gastroesophageal reflux disease (GERD). It is widely used in paediatrics [1], where it is administered orally or intravenously [2]. Oral preparations are licensed for treatment of peptic ulcer in children above 3 years, but the injection is not. [3]. As part of an ongoing research programme we are currently employing population pharmacokinetics (pop PK) analysis of sparse data to gain knowledge of the PK profiles of several drugs which are routinely used in an off-label or unlicensed manner for the treatment of children. Ranitidine is one of the drugs under investigation. Such unlicensed and off label use of drugs in paediatric patients is of great concern [4–6]. Recently, as a part of our research programme, our group has published work on analytical methodology involving drugs like metronidazole, canrenone and spironolactone [7,8] in plasma and/or dried blood spot (DBS) samples.

Conventionally, blood plasma has been used for the determination of drugs to study the pharmacokinetics (PK). Recently Emmons and Rowland [9] have discussed issues with regards to the use of DBS sampling to study pharmacokinetics. A number of analytical methods have been reported for the determination of ranitidine from various matrices and different detection techniques in the past decade. Quantitation of ranitidine has been accomplished by high performance liquid chromatography with UV and DAD detection [10–17], LC–MS [18], LC–MS/MS [19,20], derivatisation followed by fluorescence detection [21], in biological matrices i.e. human plasma [10–15,17,20], serum [16], paediatric plasma [12,15,19]. Sample clean up techniques like liquid:liquid extraction [10,11,13,17,19–21], SPE [8,12,14,15] and solid phase microextraction [18] have been reported.

The DBS technique is an alternate method of sampling which is particularly useful in neonates and infants, in whom, for clinical and ethical reasons, larger volume samples are not appropriate. The DBS technique was first established for the screening of in-born errors of metabolism in infants [22]. The technique is relatively less invasive than venous sampling, requires minimal training, can be used in a range of settings (including home sampling) and samples can be easily stored and shipped in the dried form [23,24]. Bioanalytical methods involving DBS have been reported in paediatric population [7,8,25] including our groups' work. Several bioanalytical methods in DBS have been published to quantify drugs of which a few are listed, antiretroviral drugs [26,27], everolimus [28], dextromethorphan [29] acetaminophen [30] and 25 hydroxide vitamin D_2 and D_3 [31].

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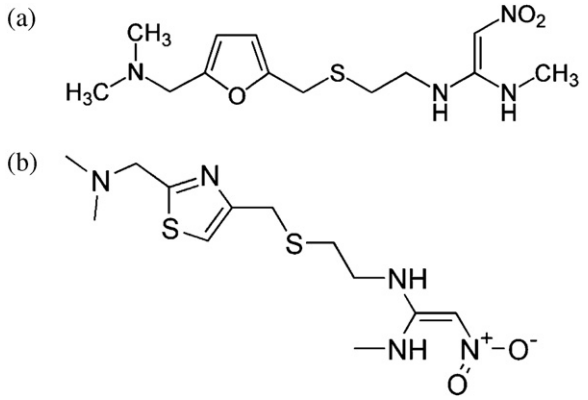

Fig. 1. (a) Ranitidine and (b) nizatidine (IS).

In the present research, we have investigated the application of LC–MS/MS method to determine ranitidine in the DBS samples in paediatric samples. This is the first report describing the quantification of rantidine in this matrix.

2. Experimental

2.1. Chemicals and reagents

All the reagents and chemicals were of analytical grade except where otherwise stated. Ranitidine HCl and nizatidine the internal standard (IS) (Fig. 1b), were purchased from Sigma (Poole, UK). HPLC grade solvent methanol and ammonium acetate buffer were purchased from BDH (Poole, UK). Oasis® HLB cartridges (1 cc/30 mg) were purchased from Waters (Dublin, Ireland). HPLC grade water was obtained using Millipore Direct-Q™ water system (Millipore, Watford, UK). Blank blood was collected from healthy human volunteers using procedures which are approved by the School of Pharmacy Ethical Committee.

2.2. Equipment

Guthrie cards (Whatman 903) and the storage pouches were purchased from RR Donnelley (Oldham, England). A Sole 6 mm punch (model PF35A0G1) was used to cut out the DBS was purchased from Rapesco (Sevenoaks, UK). The solid phase extraction was carried out manually using Waters SPE manifold (Dublin, Ireland). Solvent evaporation was performed using a Zymark Turbo Vap® LV Evaporator workstation (Zymark, Runcorn, UK).

2.3. Instrumentation

The chromatographic separation was performed using an XBridge™ C18 column (3.5 μm, 4.6 × 100 mm) preceded by an XBridge (3.5 μm, 4.6 × 20 mm) guard column of matching chemistry, both maintained at 30 °C. The LC–MS/MS system consisted of a Waters Alliance HT system 2795 separation module coupled with Waters Micromass Quattro Premier™ tandem quadrupole mass spectrometer (Waters, Manchester, UK). The mobile phase consisted of 10 mM ammonium acetate/methanol (98:2 v/v) with a flow rate of 0.3 mL/min. The MS conditions were optimised using electrospray positive ion mode (ESI+) as follows: Cone gas (nitrogen) and the desolvation gas (nitrogen) flow were maintained at 300 and 800 L/h respectively; source temperature 125 °C, desolvation temperature 350 °C; capillary voltage 2.94 V. Argon gas was used as the collision gas. Multiple reaction monitoring (MRM) transitions were selected for quantification as follows: for rantidine the quadrupole 1 was set to transmit the molecular ion *m/z* 315.45 and quadrupole 2 to transmit the product ions *m/z* 176.0 and 129.9. The cone voltage was set at 25.0 V for both the product ions and the collision energy was set at 17.0 and 25.0 eV respectively. For the IS the quadrupole 1 was set to transmit the molecular ion *m/z* 332.1 and the quadrupole 2 to transmit the product ion *m/z* 154.8, where in the cone voltage was set to 30.0 V and the collision energy was set to 20.0 eV. The dwell time for each ion was set at 0.05 s.

2.4. Software

The 2795 LC system and the Quattro Premier mass spectrometer were both controlled by MassLynxTM 4.0 Software and the QuanLynx Application Manager was used to process the data and quantification.

2.5. Preparation of standard stock and working solutions

Ranitidine, nizatidine (IS) stock solutions (1 mg/mL) and the respective working standard solutions were prepared in methanol. Ranitidine working standards at concentrations 25, 10, 5, 2.5, 1.25, and 0.5 μg/mL were prepared for calibration standards and used to spike the whole blood. Similarly ranitidine working standards at concentrations 25, 22.5, 8, 1 and 0.5 μg/mL were prepared separately for QC standards. Nizatidine working standard was prepared at the concentration 25 and 10 μg/mL. All the solutions were stored at 4 °C and brought to room temperature (20 °C) before use.

2.5.1. Preparation of calibration standards and quality control (QC) samples in whole blood

A set of calibration standards (10–500 ng/mL) in whole blood was constructed by spiking 980 μL of whole blood with 20 μL of appropriate ranitidine working standard solution containing 0.5, 1.25, 2.5, 5.0, 10.0 and 25.0 μg/mL, to give final concentration at 10, 25, 50, 100, 200 and 500 ng/mL. The QC samples at concentration 10 ng/mL (LOQ), 20 ng/mL (low QC), 160 ng/mL (middle QC), 450 ng/mL (high QC) and 500 ng/mL (ULOQ) were prepared separately in similar manner.

2.6. Blood spotting

Spiked blood was kept at room temperature for 30 min before spotting, to allow for the equilibration of ranitidine in the sample. The DBS were prepared by spotting 30 μL of the spiked blood from both calibration and QC standards onto a Guthrie card using a calibrated pipette. The samples were allowed to dry at room temperature in the dark (cupboard) for at least 3 h before storing at −20 °C until analysis. This was done purely to avoid any physical contamination (if left on the laboratory bench or in an open area) and not because of any stability issue. These calibration and QC standards were used in method validation.

2.7. Sample preparation, extraction and analysis for DBS

A 6 mm disc was punched from the centre of the DBS into a clean tube. This was then extracted by addition of 1 mL methanol containing 100 ng of the IS and vortex mixed for 5 min. The methanolic extract was then evaporated to dryness under nitrogen at 37 °C using Zymark Turbovap and reconstituted with 1 mL of water. The contents were mixed for 3 min and further subjected to the SPE clean up using Oasis® HLB cartridges. The cartridges were conditioned with methanol (1 mL) and water (1 mL) following which the above DBS water extract (1 mL) was loaded, washed with 5% methanol and then eluted with 1 mL of methanol. The eluate was evaporated to dryness at 37 °C and reconstituted in 50 μL of methanol. A volume of 5 μL was injected on LC–MS/MS.

2.8. Validation procedures

All validation experiments in DBS were performed according to the FDA guidelines [32].

2.8.1. Selectivity

The selectivity of the developed method was determined by analysing blank DBS samples from six different sources. The matrix effect was studied for these samples as they were being analysed using LC–MS/MS. This is discussed in Section 2.8.5.

2.8.2. Linearity

Linearity of the method was assessed using five calibration curves analysed on five consecutive days. The peak area ratios (response) against the respective analyte concentration were used to assess the relationship between the response and concentration. The calibration curves were fitted by least squares linear regression using $(1/X^2)$ as the weighting factor to calculate slopes, intercept and correlation (r).

2.8.3. Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of the intercept (σ) and the slope (S) of calibration curves as follows:

$$\text{LOD} = 3.3(\sigma/S) \text{ and LOQ} = 10(\sigma/S)$$

2.8.4. Accuracy and precision

The determination of the accuracy and precision of the assay in DBS was accomplished by the analysis of five replicate sets of each of the 5 concentrations (10, 20, 160, 450 and 500 ng/mL) of the QC samples on five separate occasions. The accuracy was calculated by comparing the measured concentration with the nominal (true) concentration as the mean percent relative error (% RE). The precision of the methods was expressed as the relative standard deviation (% RSD).

2.8.5. Matrix effect and recovery

The matrix effect (influence of matrix on electrospray ionisation) was studied and the quantification of ranitidine and IS was monitored using quantitative methods [33] at three concentrations (10, 160 and 500 ng/mL). This involved a comparison of:

(a) The instrument response for the calibrators (including the IS) injected directly in mobile phase (neat solutions).
(b) The same amount of compound added to extracted blank samples (post-extraction spiked samples).
(c) The same amount of analyte added to the biological matrix before extraction (pre-extraction spiked samples).

The matrix effect was measured by comparing the responses from post-extraction spiked samples with those from pure solutions; if the ratio is <85% or >115%, an exogenous matrix effect is implied [33].

$$\%\text{Matrix effect} = \frac{\text{response (post extraction spiked sample)}}{\text{response (neat solution)}} \times 100$$

Recovery was determined by comparing the MS response of the pre-extracted spiked samples with those spiked post-extraction into a blank matrix. Because both samples have the same matrix the matrix effect could be considered the same for pre- and post-extraction spiked samples.

$$\%\text{Recovery} = \frac{\text{response (pre extraction spiked sample)}}{\text{response (post extraction spiked sample)}} \times 100$$

Overall process efficiency was calculated by comparing the responses from pre-extraction spiked samples with those from neat solutions.

$$\%\text{Process efficiency} = \frac{\text{response (pre extraction spiked sample)}}{\text{response (neat solution)}} \times 100$$

2.9. Stability

2.9.1. Stock solutions

The stock solution stability for both ranitidine and the IS was tested at the selected storage temperature of 4 °C after a period of one and six months, by comparing with those of freshly prepared stock solution. Stability was also performed at room temperature (20 °C) for up to 24 h.

2.9.2. DBS samples

Stability of the spiked DBS samples stored at room temperature and −20 °C was assessed over a period of 24 h, one and six months. This was done by extracting freshly spiked DBS samples and comparing the ratio of peak areas (ranitidine to IS) with those obtained for stored samples.

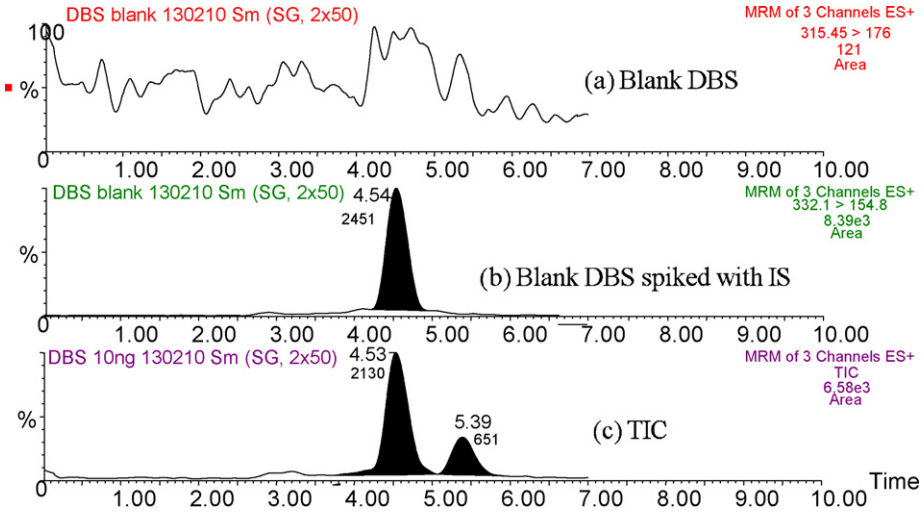

Fig. 2. Chromatograms of (a) blank DBS, MRM of ranitidine at m/z 176.0, (b) blank DBS spiked with the IS, MRM of IS at m/z 154.8 and (c) total ion chromatogram (TIC) of blank DBS spiked with ranitidine at the LOQ (10 ng/mL) and IS (100 ng/mL).

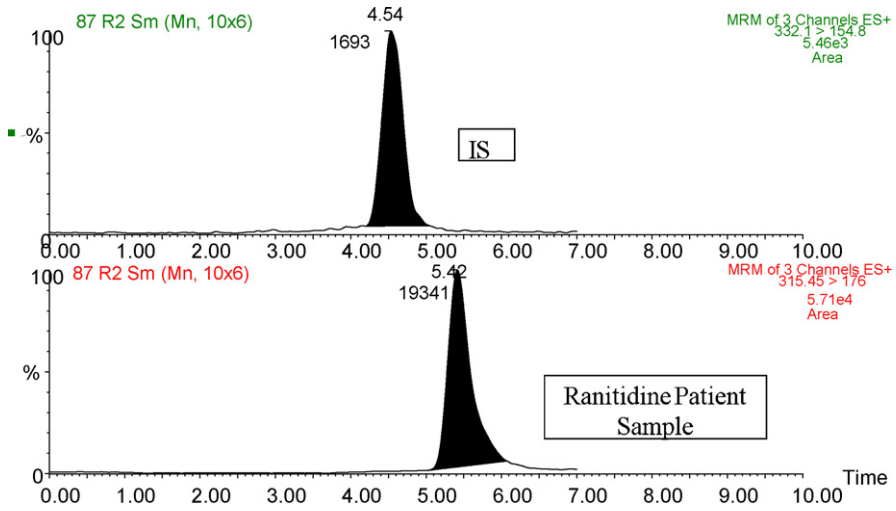

Fig. 3. Chromatogram of a paediatric patient of age 7 years, 2 months, weighing 30.5 kg, administered orally a total dose of 150 mg of ranitidine. Concentration of ranitidine measured 315 ng/mL.

3. Results and discussion

3.1. Method development

In the early stage of method development a short HPLC column (XBridge 3.5 μm, 2.1 × 30 mm) was used using the same mobile phase as mentioned in Section 2.3 and the flow rate was kept at 0.2 mL/min. The retention time was 1.21 min for both ranitidine and IS. There was no matrix effect seen but the results were not reproducible probably due to the co-elution of both the analyte and the IS. Hence a longer column was used, using the same mobile phase at a flow rate 0.3 mL/min. The results were reproducible and the chromatogram (Fig. 2) showed a good resolution between ranitidine and IS. The method was validated using these new chromatographic conditions. The use of stable-isotope labelled internal standards is desirable in MS-based assays as this has a very positive influence in reducing the variability of the assay due to matrix effects. This is not always possible either due to the high costs involved in synthesising these compounds or due to non availability. Although ranitidine labelled isotope was available in the market when we started our work the cost was beyond the budget of this project. Nizatidine was chosen as an IS because of its structural and chemical similarity to ranitidine. There was no matrix effect seen as discussed in Section 3.3.4 and the overall recovery (process efficiency) was good. This method was used to quantify ranitidine from the paediatric patient samples as described in Section 4. Fig. 3 represents the chromatogram of a paediatric patient of age 7 years, 2 months, weighing 30.5 kg, who was administered orally a total dose of 150 mg of ranitidine, representing a concentration of 315 ng/mL. Based on the DBS matched plasma patient data, the DBS concentration were estimated and for most of the samples were found to be above the LOQ. Hence the sensitivity of the assay was suitable for the analysis.

Unlike plasma, the DBS poses a significant challenge in extraction and analysis due to various endogenous substances in the whole blood and impurities in the paper. This can cause matrix effect, hence a sample clean up step such as SPE was introduced during the extraction process. There have been reports in the literature [25,26,28–30] wherein the extracted samples have been injected straight into the LC–MS/MS without any sample clean up. In our own work we found that there was a significant reduction of the interfering substances and hence a reduction in the matrix effect when SPE was applied. Also, Fig. 4 clearly shows that the sensitivity of ranitidine increased 2 fold after SPE.

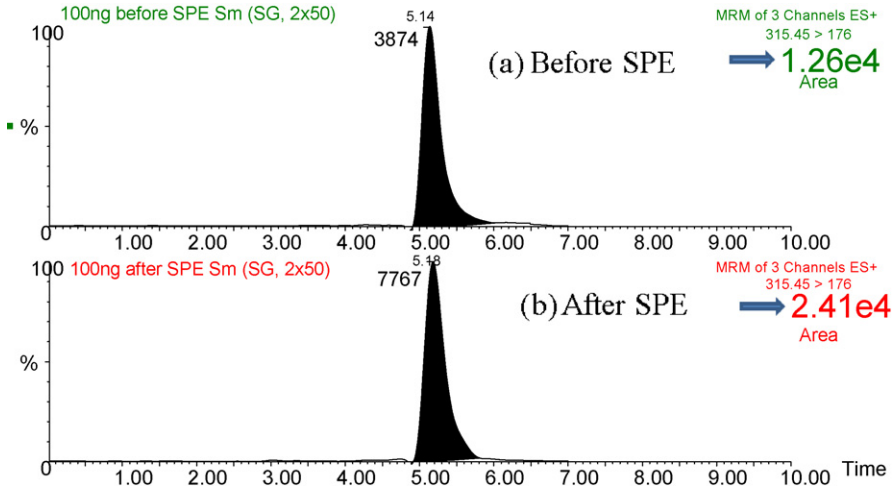

Fig. 4. Chromatograms of the ranitidine spiked at 100 ng/mL (a) before the SPE and (b) after the SPE clean up.

Table 1
The intra and inter day precision and accuracy for ranitidine ($n = 5$) in DBS.

Concentration (ng/mL)	Precision		Accuracy
	Mean ± SD	RSD %	
Intra-day			
10	10.6 ± 0.6	5.9	106
20	20.8 ± 1.1	5.1	104
160	160 ± 8.6	5.4	100
450	468 ± 8.2	1.8	104
500	509 ± 9.0	1.8	102
Inter-day			
10	9.74 ± 0.1	0.9	96.7
20	20.6 ± 0.4	1.8	103
160	170 ± 5.4	3.2	106
450	462 ± 9.6	2.1	103
500	529 ± 32.0	6.1	106

3.2. Blood spotting procedure

The weights of discs ($n = 10$) punched from the Guthrie cards after drying for 3 h, for both blank and non-spiked whole blood, showed excellent reproducibility – blank 5.17 ± 0.17 mg (RSD, 2.49%); non-spiked whole blood 7.78 ± 0.34 mg (RSD, 4.43%) confirming the suitability of the drying process and the precision of the disc cutting procedure. In a previous study [8] our group has shown that the volume (20–100 μL) of drug-spiked blood applied to a Guthrie card does not result in any significant variation in estimated concentration when the same disc size is used for determination of the drug concentration.

3.3. Method validation

3.3.1. Selectivity

The selectivity of the method was established by the analysis of blank DBS spiked at the LOQ (10 ng/mL) and IS (100 ng/mL) as shown in Fig. 2. No significant interferences and matrix effect was observed at the retention times of rantidine and IS in spiked and patient DBS samples.

3.3.2. Linearity

Calibration plots of ranitidine/IS peak area ratios versus the nominal concentration of ranitidine in DBS were constructed and a weighted $1/X^2$ linear regression applied to the data. The back calculated concentrations for each calibration points expressed as a relative error were between 4.2 and 7.2% over the calibration range. Quanlynx application manager was used to perform the statistical analysis. Linear response was observed over the range 10–500 ng/mL with a mean $r = 0.996 \pm 0.002$ ($n = 5$). The mean slope and intercept values from the calibration curves were 0.0283 and −0.0176 respectively. The LOQ was calculated at 10 ng/mL.

3.3.3. Accuracy and precision

Inter and intra-day data for accuracy and precision in DBS are given in Table 1. All the values obtained were well within the guidelines published by the FDA [32]. Intra-day and inter-day variations were established by analysing samples ($n = 5$) at five different concentrations 10, 20, 160, 450 and 500 ng/mL, on 5 separate days. The inter-day precision for the QCs 10, 20, 160, 450 and 500 ng/mL was between 0.9 and 6.1% and the accuracy was well within ±15%. Thus the method possessed good accuracy and precision and was suitable for use in the analysis of the clinical samples.

3.3.4. Matrix effect and recovery

Co-eluting, matrix components may reduce or enhance the intensity of the signals of the analytes and affect the reproducibility and accuracy of a bioanalytical method which might eventually affect the integrity of the pharmacokinetic (PK) data for which the method was designed. The US FDA guidelines [32] clearly indicate the need for the assessment of the matrix effect during the development and validation of LC–MS/MS methods to ensure that the precision, selectivity and sensitivity are not compromised. Based on the quantitative method, the estimated matrix effect (% ME) on both ranitidine and IS was found to be within 85% and 115%. This indicates the lack of any major ion suppression on enhancement for the analytes in this method. The overall recovery of ranitidine at three QC concentrations (10, 160 and 500 ng/mL) was 92 ± 1.5% and 87 ± 2.1% for the IS in DBS.

Table 2
Stability of ranitidine in DBS ($n = 3$) under various storage conditions.

QC (ng/mL)	Remaining percentage[a] (mean ± SD)		
	24 h (20 °C)	1 month (−20 °C)	6 months (−20 °C)
10	94.3 ± 2.0	93.3 ± 6.6	92.3 ± 2.5
160	96.6 ± 1.5	96.6 ± 2.0	95.6 ± 3.0
500	97.6 ± 3.5	96.3 ± 3.0	96.6 ± 4.7

[a] Remaining percentage = (concentration found)/(concentration added) × 100.

3.4. Stability

3.4.1. Stock solution

The ranitidine stock solution was stable in methanol at 4 °C for a period of six months and at room temperature for 24 h.

3.4.2. DBS samples

Assay of stability samples (Table 2) of the spiked DBS stability samples showed values comparable with freshly prepared samples at room temperature and −20 °C for the period of 24 h, one month and up to 6 months.

4. Clinical application

The above validated DBS method was applied to analyse the paediatric clinical samples. A total of 81 DBS samples from 36 patients were analysed. The study protocol was approved by the Ethics Committee of the School of Medicine/Queen's University Belfast. Eligible patients (children up to 12 years of age) were those who had been hospitalised and given ranitidine in an off label and unlicensed manner as a part of their clinical management. Informed verbal and written consent was obtained from the parent(s) or guardian(s) of all eligible patients. Assent was also obtained from paediatric patients who were 8 years or older. Samples were collected from each patient at times when other blood samples were being withdrawn for routine laboratory analysis (opportunistic sampling). The patient blood sample was collected in blood collection tubes S-MONOVETTE® (Sarstedt, Germany). An aliquot of blood 30 μL was spotted onto a Guthrie card and allowed to dry for 3 h in the dark at ambient temperature.

No difficulties were identified during the analysis. All the values obtained from the patient sample analysis were within the calibration range except for a few samples whose concentrations were found below LOQ. This was due to the sparse sampling procedure that was adopted in which samples were obtained from patients when blood samples were being taken for routine clinical measurements and not when decided by researchers. Fig. 5 represents a plot of rantidine–DBS concentration versus time, where eight samples were collected opportunistically from the same patient over a period of time.

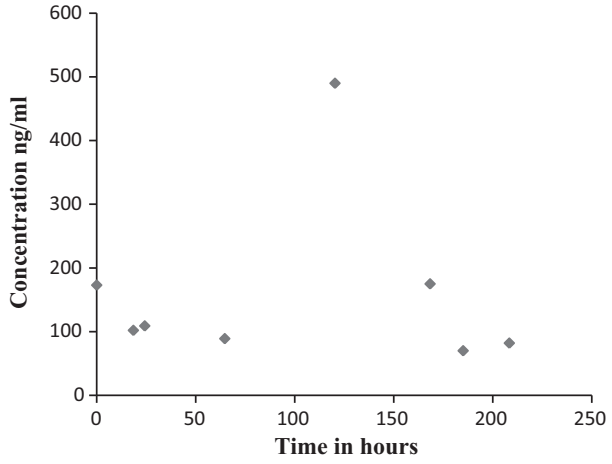

Fig. 5. Profile of a patient (6 years, 23 kg) administered ranitidine (75 mg) orally twice daily. DBS sample taken opportunistically over a period of 8 days. The first time point (0 h) is the first sample taken.

5. Conclusion

This is the first report in the literature describing the quantitative estimation of ranitidine in small volume paediatric samples using the dried blood spots (DBS) approach. A simple and rapid bioanalytical assay has been developed and validated in DBS using LC–MS/MS. The validated method in DBS was demonstrated to be accurate, precise and complied with the regulatory guidelines. The method was found to be selective and sensitive enough to extract and quantify ranitidine in 6 mm DBS disc containing approximately 11 μL of whole blood. The sample clean up step was included to enhance the sensitivity of the assay. The stability of ranitidine in DBS provides reassurance that there is no degradation of the drug from the time of spotting to a period of six months at −20 °C and at room temperature for 24 h. In the present research the blood samples from the patients were collected via venous cannula and spotted onto the Guthrie cards. DBS sampling can also involve obtaining blood samples from a finger or heel prick, as demonstrated by our group in recent publications [7,8]. The analytical data for all the patients participating in the study have been subjected to a population PK evaluation using non-linear mixed effect modelling (NONMEM®). The plasma versus DBS correlation data and pop PK data will be published elsewhere.

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