4

Dried blood spot analysis for therapeutic drug monitoring of pazopanib

Djoeke de Wit, Jan den Hartigh, Hans Gelderblom, Yanwen Qian, Margret den Hollander, Henk Verheul, Henk-Jan Guchelaar and Nielka P. van Erp

ABSTRACT

Background Dried blood spot (DBS) sampling is potentially a more patient-friendly and flexible alternative to venous sampling of pazopanib. This study determines the agreement between pazopanib DBS- and plasma concentrations to facilitate implementation of pazopanib DBS sampling into clinical practice.

Patients and Methods Paired DBS and plasma samples were collected in 12 patients. Pazopanib plasma concentrations were calculated from DBS concentrations using the formula: plasma concentration = $DBS_{concentration}$/(1-haematocrit). Passing-Bablok and Bland-Altman analyses were used to determine the agreement between calculated and measured plasma concentrations. We predefined a clinical acceptance limit of 25% for the Bland-Altman analysis.

Results Passing-Bablok analysis showed a small constant (intercept estimate -8.53(95%-CI; -12.22 to -4.41)) and slightly proportional (slope estimate 1.15 (95%-CI; 1.04-1.24)) bias between calculated and measured concentrations. This bias was clinically non-relevant as shown by Bland-Altman analysis; the mean ratio of calculated to measured concentrations was 0.94 (95%-CI; 0.65-1.23). The clinical acceptance limits were well within these 95% limits of agreement. More specifically, 92.6% of the data points were within the predefined acceptance limits.

Conclusion Pazopanib plasma concentrations can be accurately calculated from DBS concentrations. Although validation of DBS cards prepared by patients themselves is required, these results show that DBS sampling can be used to monitor pazopanib therapy in clinical practice.

Journal of Clinical Pharmacology 2015, Epub ahead of print

Introduction

Pazopanib hydrochloride (Votrient®) is an oral multi-targeted tyrosine kinase inhibitor (TKI) used for the treatment of metastatic renal cell carcinoma (mRCC) and metastatic soft tissue sarcoma (STS) [1,2]. For patients with mRCC, a correlation between pazopanib exposure and treatment outcome has been demonstrated [3]. Suttle et al. showed that a higher treatment response was seen for patients with a C_{trough} level > 20.5 mg/L and a higher incidence of toxicity was seen in patients with a C_{trough} level > 36 mg/L. This implies that the optimal therapeutic window for pazopanib in patients with mRCC lies between a C_{trough} level of 20.5 - 36 mg/L. There is a large variability in pazopanib exposure between patients (40-60 CV% in AUC) leading to the risk of sub- or supra-therapeutic C_{trough} levels and therefore to decreased therapeutic effects or more toxicity [3,4].

Therapeutic drug monitoring (TDM) can be useful to reach concentrations within the therapeutic window in order to optimize the efficacy and minimize the toxicity of therapy. The evidence for usefulness of TDM of pazopanib is accumulating and monitoring of C_{trough} levels is currently indicated in case of extreme or unexpected toxicity, a lack of clinical benefit, suspected drug interactions or suspected non-adherence to therapy [4-6]. In the first phase I trial, pazopanib exposure increased as the dose increased, although a plateau at 800 mg once daily was seen [7]. Theoretically, this might introduce problems when a patient has an exposure below the threshold for efficacy and needs an increased dose. However, the finding of a plateau is based on a limited number of patients and therefore not conclusive yet. In addition, results from two pazopanib TDM study suggest that exposure does increase with doses above 800 mg [4,8].

At present, pazopanib concentrations are monitored in plasma collected by venous sampling [9]. However, sampling by venipuncture has several disadvantages including its invasive character, the requirement for patients to travel to the clinic and the need for trained personnel. Compared to venous sampling, dried blood spot (DBS) sampling is a convenient, simple, flexible and more patient friendly alternative to collect blood in an at home setting. With clear instructions and after adequate training, patients should be able to self-collect DBS samples. The added value and feasibility of DBS collection for TDM has been shown effective for several drugs including anti-epileptics, immunosuppressants and antiretroviral drugs [10-12].

Here, we describe the results of a study investigating the feasibility of DBS for TDM of pazopanib. The objective of this study is to determine the agreement between pazopanib DBS- and plasma concentrations in order to facilitate the future implementation of pazopanib DBS sampling into clinical practice.

Methods

Patients

The collection and analysis of DBS and plasma samples was part of a larger phase I study that investigated the feasibility of TDM for dose individualization of pazopanib [4]. Included patients were ≥ 18 years with progressive disease from an advanced solid tumor, a WHO performance status ≤ 2 that had no standard treatment options available. All patients had adequate haematologic, renal and liver function reserves. The study was approved by the institutional ethics committee (Leiden University Medical Center, The Netherlands) and all patients gave written informed consent before entering the study. Between July 2012 and June 2013, 13 patients were included of whom 12 also participated in the DBS part of the study

Sampling

At day 14 of standard 800 mg pazopanib therapy, patients were admitted to the hospital for pharmacokinetic sampling. EDTA-blood samples were collected by venepuncture pre-dose and 1, 2, 3, 4, 6, 8, 10 and 24 hours after pazopanib intake. From these EDTA blood samples, 15 µL blood was collected into an EDTA capillary tube and spotted onto a pre-marked circle on a Whatman FTA® DBS card. This procedure was repeated 2 times to fill the 3 pre-marked circles on the card. After spotting the DBS cards, venous blood samples were centrifuged at 3,000 rpm for 5 minutes; the supernatant plasma was stored at -20°C until the day of analysis.

In addition to the DBS sampling cards prepared with venous blood, DBS sampling cards prepared by finger prick were collected pre-dose, and 3 and 8 hours after pazopanib intake. After disinfection of the skin with alcohol 70%, a lancet puncture was performed. The first drop of blood was discarded, thereafter 15 µL blood from the finger was collected using the above described capillary tube and spotted onto the DBS card. This procedure was repeated 2 times to fill the 3 pre-marked circles on the card

After drying for at least 2 hours, DBS cards were stored at room temperature in a closed plastic bag containing 2 sachets of desiccant. Thereafter, finger prick DBS cards (n = 3), venous DBS cards (n = 9) and plasma samples (n = 9) were all sent to GlaxoSmithKline, USA for further bio-analytical analysis.

Analysis

For the analysis of DBS pazopanib concentrations, a 4 mm diameter disc was punched out from the 15 µL dried blood spot. Per subject only 1 blood spot out of 3 was analyzed. Pazopanib was extracted from this disc with the use of 50 µL formic acid and 400 µL methanol containing an isotopic labelled internal standard, [$^{2}H_{3}$ ^{13}C]-pazopanib. After through mixing and centrifugation, 200 µL of the extract was taken into an auto-sample

tube where it was diluted with 200 µL of water before injection onto a HPLC-MS/MS system for analysis. This validated method was linear within the concentration range of 0.1-50 µg/mL pazopanib. The within- and between-run imprecisions were ≤ 11.4% and ≤ 6.2% respectively and the accuracy of this method was between -10.5% and 5.5%. Samples were stable on the DBS card for at least 75 days at ambient temperature. There was no influence of haematocrit levels (0.2 to 0.65) on the performance of this assay.

For analysis of pazopanib concentrations in plasma, 20 µL of plasma was extracted by adding 500 µL of acetonitrile/10 mM ammonium acetate (80/20 v/v) containing 100 ng/mL of [$^{2}H_{3}$ ^{13}C]-pazopanib as the internal standard. This was followed by vortex mixing and centrifugation at approximately 6200 g for 20 minutes. The supernatant was transferred into clean tubes and injected onto a HPLC-MS/MS system for analysis. This validated method was linear within the concentration range of 0.1 - 50 µg/mL pazopanib. The within- and between-run imprecisions were ≤ 14.7% and ≤ 2.9% respectively and the accuracy of this method was between -4.3% and 5.5%. Samples were stable in plasma for at least 530 days at -20 °C and 24 hours at ambient temperature.

Calculation of plasma concentration

Pazopanib plasma concentrations were calculated from DBS concentrations using the previously described formula: plasma concentration = $DBS_{concentration}$ /(1-haematocrit) [13]. The blood:plasma ratio of pazopanib ranges from 0.59 to 0.93 which suggests only a minimal association of pazopanib with blood cells [14]. In addition, only the unbound fraction of a drug can partition into blood cells [15]. Since pazopanib has a high protein binding of > 99.9%, the unbound fraction will be negligible [16]. Therefore, the fraction of pazopanib bound to red blood cells (haematocrit) was ignored in the above described formula. Plasma concentrations were calculated using both patient specific measured haematocrit values and fixed haematocrit values of 0.40 and 0.45 for males and females, respectively. A paired Student's t-test was used to test for a difference in calculated plasma concentrations using measured and fixed haematocrit values.

Statistics

Passing-Bablok regression and Bland-Altman analysis were used to determine the agreement between the two sampling methods [17,18]. Passing-Bablok regression analysis tests for a constant bias and proportional bias between two methods. If the 95%-CI for the intercept of the regression line includes 0, no constant bias is observed. If the 95%-CI for the slope of regression line includes 1, there is no proportional bias between the two tested methods. We used Bland-Altman analysis to define the

clinical relevance of any found bias. As suggested by Bland and Altman, a clinical and practical acceptance limit for the found ratio was determined [18]. A 25% range around the found ratio of the two methods was determined to be clinically and practically relevant since pazopanib can only be dose adjusted in steps of 25% of the total dose (200 mg tablets are the lowest dose available). Hence, the difference should be > 25% to result in a possibility to adjust the dose.

Analysis was performed with Microsoft office Excel (Microsoft Inc, Redmond, WA) and add-in Analyse-it statistics software (Analyse-it Software, Ltd, Leeds, UK).

Results

Patients

Between July 2012 and June 2013, 12 patients were enrolled in this DBS study. Characteristics of the patients included are summarized in Table 1.

Agreement between DBS concentrations prepared by finger prick and with venous blood

Concentrations measured at the same time points in DBS samples prepared by finger prick and prepared with venous blood were in good agreement with each other (Figure 1A). Passing-Bablok regression showed that that there was no constant (intercept estimate -0.71 (95%-CI; -3.41 to 2.23)) or proportional bias (slope estimate 1.05 (95%-CI; 0.93 to 1.17)) between the two sampling methods for the preparation of DBS cards. In this study, we collected 3 DBS cards prepared by finger prick per patient compared to 9 DBS cards prepared with venous blood. Since both methods for DBS card preparation were in agreement with each other and values therefore interchangeable, we used the (more extensive) data from the venous DBS cards for all further described analysis.

DBS vs. plasma concentrations

Pazopanib DBS concentrations (uncorrected concentrations in blood) were on average 48.0% (SD 8.5%) lower than measured plasma concentrations (Figure 1B). Passing-Bablok regression analysis showed that there was a constant (intercept estimate -4.68 (95%-CI; -6.48 to -2.47)) and proportional bias (slope estimate 0.63 (95%-CI; 0.57 to 0.68)).

Calculated vs. measured plasma concentrations – Passing-Bablok analysis

Calculated plasma concentrations using patient specific haematocrit values were on average 94.0% of measured plasma concentrations. Variability was relatively large (SD 14.7%, range 61.6% - 134.9%). Using a fixed haematocrit value, calculated plasma concentrations were on

Table 1 Patient baseline characteristics

Characteristic	
N	12
Age (years)	48 (23 - 68)
Sex (n)	
Male	11 (92%)
Female	1 (8%)
Length (cm)	183 (168 - 186)
Weight (kg)	90 (71 - 101)
ECOG PS (n)	
0	3 (25%)
1	9 (75%)
Hematology	
Haematocrit	0.45 (0.40 - 0.49)
ANC (× 10^9/L)	4.12 (2.49 - 6.42)
Platelets (× 10^9/L)	261 (150 - 394)
Hemoglobin (mmol/L)	9.05 (6.9 - 9.9)
Chemistry	
AST (U/L)	23 (17 - 33)
ALT (U/L)	21 (12 - 33)
Creatinine (mg/dL)	0.85 (0.71 - 0.97)
Total bilirubin (mg/dL)	0.52 (0.35 - 0.70)
Blood pressure (mmHg)	
Systolic	129 (113 - 142)
Diastolic	79 (60 - 93)
Tumor type (n)	
Chordoma	4
Sarcoma	3
Pancreatic cancer	1
Schwannoma	2
Ganglioneuroma	1
Granulair cell tumor	1

Data are presented as median (range) unless stated otherwise. Abbreviations: ALT, alanine aminotransferase; ANC, absolute neutrophil count; AST, aspartate aminotransferase; ECOG PS, Eastern Cooperative Oncology Group performance score; WBC, white blood count.

average 95.0% of measured concentrations with comparable variability (SD 15.5%, range 65.0 - 144.0%). No significant differences between both approaches (measured and fixed haematocrit values) was observed (95%-CI of difference in calculated plasma concentrations; -0.19 to 0.57, $P = 0.315$).

Passing-Bablok analysis showed a small constant bias (intercept estimate -8.53 (95%-CI; -12.22 to -4.41)) and slightly proportional bias (slope estimate 1.15 (95%-CI; 1.04 to 1.24)) between calculated and measured plasma concentration when patient specific haematocrit values were used (Figure 2). Similar results were found when a fixed haematocrit value was used; intercept estimate -9.67 (95%-CI; -13.28 to -5.51) and slope estimate 1.17 (95%-CI; 1.07 to 1.26).

Figure 1 Agreement between A) DBS concentrations obtained by finger prick and DBS concentrations obtained by venous blood. B) DBS concentrations and measured plasma concentrations

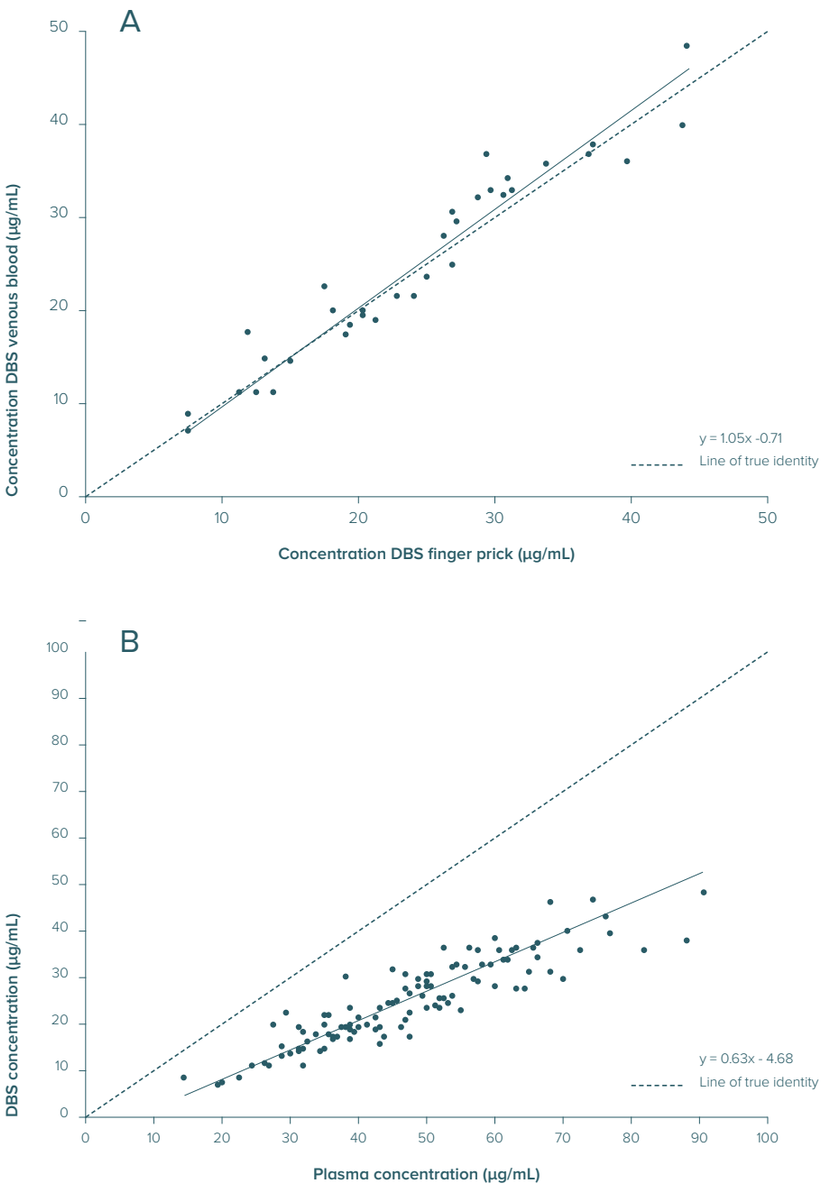
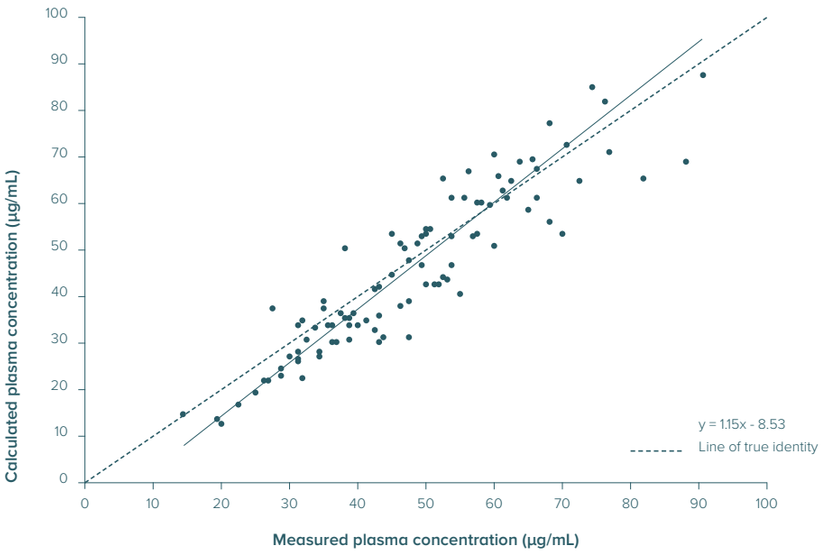


Figure 2 Agreement between measured and calculated plasma concentrations using patient specific haematocrit.

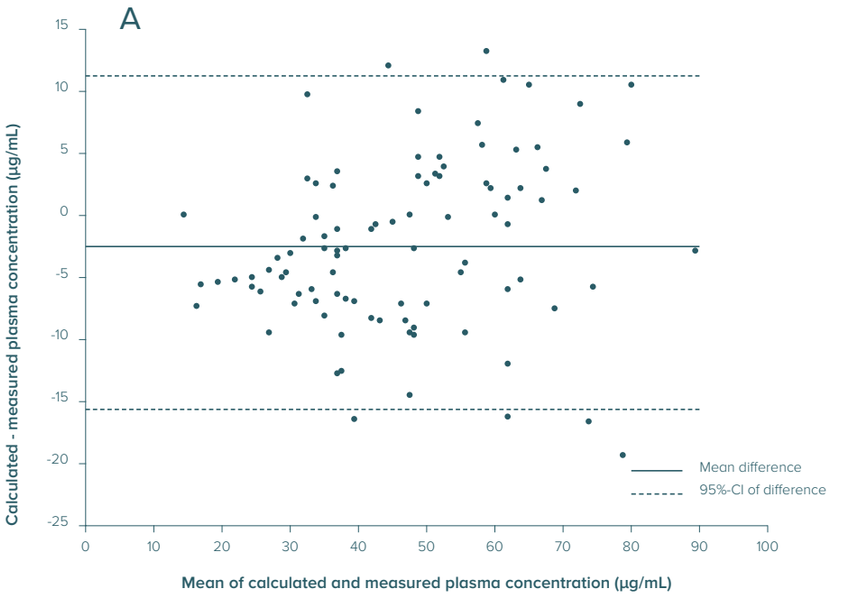


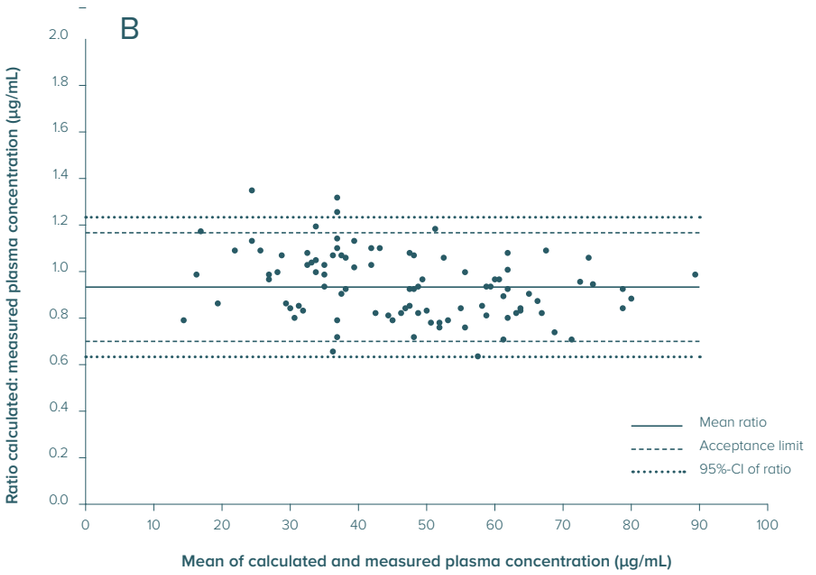
Calculated vs. measured plasma concentrations – Bland-Altman analysis

The difference in pazopanib concentrations between calculated and measured plasma concentrations using patient specific haematocrit values ranged from -19.2 to 13.2 µg/mL with a mean difference of -2.4 µg/mL (SD 6.8 µg/mL, Figure 3A). The mean ratio of calculated to measured plasma concentrations was 0.94 with the 95% limits of agreement of this ratio being 0.65 to 1.23 (Figure 3B). The clinical acceptance limits which were set at 25% around the found mean ratio, fell well within the 95% limits of agreement (0.71 to 1.18). More specifically, 92.6% (88 out of 95) of the data points were within the clinical acceptance limits.

Similar results were found when fixed haematocrit values were used. The difference ranged from -18.7 to 16.7 with a mean difference of -2.0 µg/mL (SD 7.1 µg/mL). The mean ratio of calculated to measured plasma concentrations was 0.95 with the 95% limits of agreement of this ratio being 0.65 to 1.25. The clinical acceptance limits (0.71 to 1.19) also fell well within the agreement limits. Using fixed haematocrit values, 12.6% (13 out of 103) of the data points exceeded the clinical acceptance limits.

Figure 3 Bland-Altman plot of A) difference between calculated and measured against mean plasma concentrations using patient specific haematocrit. B) ratio of calculated and measured against mean plasma concentrations using patient specific haematocrit.





Clinical relevance

A pazopanib trough concentration of 20.5 µg/mL is suggested as the threshold for efficacy in mRCC patients [3]. In Table 2, decision making based on measured and calculated plasma concentrations are compared. In 1 case (9.1%), there would have been a difference in decision making based on exposure. The measured plasma C_{trough} was 24.7 µg/mL compared to a calculated C_{trough} of 19.5 µg/mL from DBS. In all other cases, clinical decision making would have been the same based on either the measured or calculated plasma concentration. The same results were found when a fixed haematocrit value was used.

Table 2 Clinical decision making based on calculated and measured C_{trough} levels

	Calculated C_{trough} level < 20.5 µg/mL	Calculated C_{trough} level ≥ 20.5 µg/mL
measured C_{trough} level < 20.5 µg/mL	2	0
measured C_{trough} level ≥ 20.5 µg/mL	**1**	8

In **bold** a difference in clinical decision making based on calculated plasma C_{trough} levels using patient specific haematocrit and measured plasma C_{trough} levels

Discussion

The present study shows that pazopanib plasma concentrations calculated with the use of DBS, are in good agreement with actually measured pazopanib plasma concentrations. This implicates that DBS sampling can be used as an alternative sampling strategy for the determination of plasma concentrations to monitor pazopanib therapy.

A small constant, and slightly proportional bias was shown between calculated and measured pazopanib plasma concentrations. However, these biases were clinically not relevant as the vast majority of data points were within the predefined clinical acceptance limits. In addition, the difference between calculated and measured plasma concentrations would have resulted in different clinical decision making in only one out of 11 cases. It should be noted that in this case the difference between calculated and measured concentrations was small and concentrations were close the defined target of 20.5 µg/mL. Overall, these results show that DBS sampling can be used as an alternative – more patient friendly – sampling strategy to monitor pazopanib therapy in clinical practice.

Previously, Kralj et al investigated DBS sampling and analysis for the TKIs imatinib, nilotinib and dasatinib [19]. They used the same formula and also found good agreement between calculated and measured plasma concentrations. In the current study, we used both patient specific as well as fixed haematocrit values for the estimation of pazopanib plasma concentrations. The percentage of data points within the clinical acceptance limits when fixed haematocrit values were used, was slightly lower in comparison to when patient specific haematocrit values were used. However, no significant difference between calculated plasma concentrations using patient specific or fixed haematocrit levels could be shown. In addition, there was no difference in clinical decision making based on C_{trough} levels when patient specific or fixed haematocrit values were used. This indicates that fixed haematocrit values can be interchangeably used instead of measured haematocrit values for the calculation of pazopanib plasma concentrations when patient haematocrit levels are within the normal range.

The binding of pazopanib to red blood cells is thought to be limited and we did not take this into account for the calculation of pazopanib plasma concentrations. This may potentially cause bias in the calculation of plasma concentrations from DBS concentrations. However, calculated plasma concentrations from DBS were on average 6% lower than the measured concentrations. This demonstrates that the possibility of pazopanib partitioning into red blood cells is minimal since the calculated concentration would then have been higher otherwise. In addition, plasma concentrations could be readily predicted from DBS concentrations which also indicates that the uptake of pazopanib into red blood cells is small. This is also in agreement with the fact that pazopanib has a high plasma protein binding (> 99.9%) and the assumption that only the free unbound amount of a drug can participate into red blood cells.

In this study, DBS cards contained relatively high concentrations of pazopanib since samples were taken as part of rich PK-curves shortly after pazopanib intake to calculate pazopanib AUCs. As a consequence, there is only a limited number of DBS samples within the lower concentration range. Although splitting the Passing-Bablok regression into C_{trough} DBS samples and all other samples or into different concentration ranges did not change the results, it can be doubted whether the small proportional bias is caused by the fact that there are not enough samples in the lower concentration range or whether there is truly a proportional bias. In addition, this limits the amount of data on which agreement is based within the lower concentration range. Both bio-analytical assays for the determination of pazopanib in DBS and plasma were validated according to international guidelines, excluding an analytical cause.

DBS cards were prepared by the research nurse with the use of a 15 µl capillary. It can be argued that sampling by the research nurse

with a capillary does not truly reflect an at home sampling setting where patients spot themselves, which is a limitation of this study. On the other hand, previous studies with antiretroviral and immunosuppressive drugs have shown that 87.5 to 98% of the DBS samples obtained by patients were suitable for analysis [20,21]. Although these cards were prepared by blood drop and not capillary, it suggests that preparation of a DBS card by patients after a clear instruction is highly feasible. The perfect agreement between DBS cards prepared by finger prick and those prepared with whole blood shows that there is no engorgement of blood when a DBS is prepared by finger prick.

Conclusion

This study shows a good agreement between pazopanib levels measured in plasma and concentrations calculated from the corresponding DBS card. Although validation of clinical utility with DBS cards prepared by patients themselves is necessary, the results from this study show the feasibility of the use of DBS cards. With the ease and convenience of sample collection, DBS could be very useful for TDM of patients treated with pazopanib and potentially other TKIs in the future.

References

1 **Sternberg, C.N. et al** (2010) Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. J Clin Oncol. 28, 1061-1068.

2 **Van der Graaf, W.T. et al** (2012) Pazopanib for metastatic soft-tissue sarcoma (PALETTE): a randomised, double-blind, placebo-controlled phase 3 trial. Lancet. 379, 1879-1886.

3 **Suttle, A.B. et al** (2014) Relationships between pazopanib exposure and clinical safety and efficacy in patients with advanced renal cell carcinoma. Br J Cancer. 111, 1909-1916.

4 **de Wit, D. et al** (2014) Therapeutic Drug Monitoring to individualize the dosing of pazopanib: a pharmacokinetic feasibility study. Ther Drug Monit.

5 **de Wit, D. et al** (2015) Individualized dosing of tyrosine kinase inhibitors: are we there yet? Drug Discov Today. 20, 18-36.

6 **Yu, H. et al** (2014) Practical guidelines for therapeutic drug monitoring of anticancer tyrosine kinase inhibitors: focus on the pharmacokinetic targets. Clin Pharmacokinet. 53, 305-325.

7 **Hurwitz, H.I. et al** (2009) Phase I trial of pazopanib in patients with advanced cancer. Clin Cancer Res. 15, 4220-4227.

8 **Verheijen, R. et al** (2014) Individualized pharmacokinetically-guided dosing of pazopanib: a feasibility study in cancer patients. ESMO meeting 2014. Abstract 7651.

9 **van Erp, N.P. et al** (2013) A validated assay for the simultaneous quantification of six tyrosine kinase inhibitors and two active metabolites in human serum using liquid chromatography coupled with tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 937C, 33-43.

10 **Koster, R.A. et al** (2013) Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. Talanta. 115, 47-54.

11 **Shah, N.M. et al** (2013) A simple bioanalytical method for the quantification of antiepileptic drugs in dried blood spots. J Chromatogr B Analyt Technol Biomed Life Sci. 923-924, 65-73.

12 **Hoffman, J.T. et al** (2013) Determination of efavirenz in human dried blood spots by reversed-phase high-performance liquid chromatography with UV detection. Ther Drug Monit. 35, 203-208.

13 **Li, W and Tse, F.L.** (2010) Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed Chromatogr. 24, 49-65.

14 **Deng, Y. et al** (2013) Bioavailability, metabolism and disposition of oral pazopanib in patients with advanced cancer. Xenobiotica. 43, 443-453.

15 **Hinderling, P.H.** (1997) Red blood cells: a neglected compartment in pharmacokinetics and pharmacodynamics. Pharmacol Rev. 49, 279-295.

16 **Hamberg, P. et al** (2010) (Pre-)clinical pharmacology and activity of pazopanib, a novel multikinase angiogenesis inhibitor. Oncologist. 15, 539-547.

17 **Bablok, W. and Passing, H.** (1985) Application of statistical procedures in analytical instrument testing. J Automat Chem. 7, 74-79.

18 **Bland, J.M. and Altman, D.G.** (1986) Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1, 307-310.

19 **Kralj, E. et al** (2012) Simultaneous measurement of imatinib, nilotinib and dasatinib in dried blood spot by ultra high performance liquid chromatography tandem mass spectrometry. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 903, -156.

20 **Cheung, C.Y. et al** (2008) Dried blood spot measurement: application in tacrolimus monitoring using limited sampling strategy and abbreviated AUC estimation. Transpl Int. 21, 140-145.

21 **Kromdijk, W. et al** (2013) Therapeutic drug monitoring of antiretroviral drugs at home using dried blood spots: a proof-of-concept study. Antivir Ther. 18, 821-825.