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# Simultaneous measurement of imatinib, nilotinib and dasatinib in dried blood spot by ultra high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Imatinib, dasatinib and nilotinib are three tyrosine kinase inhibitors currently used to treat Bcr-Abl1 positive chronic myelogenous leukaemia (CML). However, achieving maximum benefit with these drugs may require optimal dosing and adherence to therapy. In those cases, therapeutic drug monitoring (TDM) can be a useful tool in managing patients with CML. The paper presents simple and high throughput method for simultaneous determination of all three TKIs in dried blood spot (DBS) samples from CML patients. DBS samples were prepared by applying 10 μL of spiked whole blood onto an Agilent DBS cards. Whole blood spot was punched out of the card, transferred to a well in a 96-well Captiva ND Lipids filter plate. After the addition of isotopically labelled internal standard, the drug was extracted with 0.1% formic acid in methanol. The collected extract (1 μL) was injected onto a Phenomenex Kinetex 50 mm × 2.1 mm C18 column and eluted with acetonitrile gradient into a triple quadrupole ESI–MS/MS Agilent 6460 operated in positive mode. The total run time was only 2.6 min. The method was validated in terms of linearity, selectivity, specificity, accuracy, precision, absolute and relative matrix effect and stability. The effect of haematocrit (Hct) on the accurate concentration determination was also examined. The method was linear in the range of 50–5000 μg/L for imatinib and nilotinib and in the range of 2.5–250 μg/L for dasatinib, with correlation coefficient values higher than 0.997. Lower limits of quantification (LLOQ) were 50 μg/L for imatinib and nilotinib and 2.5 μg/L for dasatinib. The method proved to be accurate (% bias < 13.2) and precise (CV < 10.3%) on intra- as well as on inter-day basis. Sample matrix (% ME = 94.5–106.7) and different Hct values had no significant effect on the accuracy of measured concentrations. Samples proved to be stable whilst stored on DBS cards at room temperature or in the refrigerator; however, at 40 °C the stability of dasatinib was compromised. The method presented was successfully applied to clinical samples.



## 1. Introduction

Imatinib (Gleevec or Glivec™) was developed as the first in a row of tyrosine kinase inhibitors (TKIs) and presents revolution in cancer therapy. TKIs selectively bind to the constitutively active enzymes (in case of imatinib Bcr-Abl kinase) responsible for the activation of signal transduction cascades and consequently for uncontrolled cell proliferation, apoptosis, differentiation, and adhesion [1,2]. Today, after 11 years of its launching to clinical practice, imatinib is still the first line treatment for chronic myeloid leukaemia, Bcr-Abl1 positive (CML). Its tremendous success is based on high efficacy and progression free survival, low toxicity and convenient dosing regimen [3].

However, although some patients may experience prolonged disease control whilst on imatinib, 20–25% will eventually develop imatinib resistance [4]. Several cellular and pharmacokinetic mechanisms have been proposed to influence imatinib resistance [5]. Already in 2007 Picard et al. showed that the trough imatinib plasma levels in patients treated with standard-dose imatinib were associated with both complete cytogenetic response (CCR) and major molecular response (MMR), with a plasma threshold of 1002 ng imatinib/mL [6]. Low plasma and intracellular concentrations were associated with inadequate tyrosine kinase inhibition, which is a possible reason for Bcr-Abl kinase overexpression or its mutation development [7]. Some studies suggested that dose escalation could in certain CML patients initially treated with standard-dose imatinib even overcome imatinib resistance [8].

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Because of the wide inter-patient variability in drug exposure due to differences in pharmacokinetics of imatinib (different absorption, excretion and metabolism), optimal dose adjustment seems to be a problem [9]. Several reports have demonstrated that the $C_{min}$ level of imatinib varied greatly even in patients administrated with the same daily dose [3,6,10]. Whilst some patients may benefit from high-dose imatinib therapy, others might experience increased toxicity. Therapeutic drug monitoring (TDM) can in those cases be a useful tool for physicians managing patients with CML. Blood level testing is recommended also in cases of possible drug–drug interactions or when non-adherence to therapy is suspected [11,12].

In patients with trough imatinib plasma levels exceeding 1002 ng/mL and not responding to imatinib therapy, cellular resistance to imatinib and subsequent alternative therapy, such as second generation TKIs should be considered. Dasatinib (Sprycel™) and nilotinib (Tasigna™) are new drugs developed to treat imatinib-resistant CML; however they can also be used as the first line treatment for CML patients [13,14]. They work against the same abnormal protein targeted by imatinib, but in slightly different ways [15,16]. Both were found to be more effective in eliciting a cytogenetic or haematologic response and are supposed to be better tolerated than high-dose imatinib. Dasatinib and nilotinib are active against most of the imatinib resistant Bcr-Abl mutants [17]. Whether TDM is also beneficial for these two TKIs remains to be established, but can be anticipated considering their metabolic pathways and the drug interactions potential [18,19].

Three conventional high-throughput bioanalytical procedures for the simultaneous quantification of TKIs in patient plasma have been reported in the literature [20–22]. However, determination of their concentration in whole blood (WB) is still lacking, despite being acknowledged by the regulatory authorities to be a suitable alternative. The reason is probably that whole blood presents a complex and inhomogeneous matrix, which is inappropriate for longer storage, is difficult to work with and usually involves time consuming liquid–liquid extraction before HPLC analysis of drugs [23].

Determination of substances from dried blood spot (DBS) however is a long known method, which can overcome many of these problems. DBS is a simple sampling technique, where blood samples are collected on a filter paper, dried on air and stored until analysis. It has some other practical advantages over conventional blood or plasma sampling. Considering clinical applications, DBS offers the advantage of less invasive sampling like finger prick rather than venous cannulation and lower blood volume required, which is especially great advantage in paediatrics. Samples can be conveniently taken by patients themselves and sent to the authorised laboratory by post, since there is no risk of biohazard. Finally, DBS offers logistical and cost benefits including ease of sample handling, storage and shipment of the DBS cards at room temperature [24,25].

DBS is mostly used in pharmacokinetic studies [26], however we believe, that it can also be performed in therapeutic drug monitoring of TKIs. The present article describes development and validation of a fast and simple high-throughput LC–MS/MS method for simultaneous monitoring of imatinib, dasatinib and nilotinib in blood spot from CML patients.

## 2. Experimental

### 2.1. Chemicals and materials

Imatinib mesylate ≥98% was purchased from Sequoia Research, Pangbourne, UK. Nilotinib (base), dasatinib (base) and stable isotope labelled internal standards [$^2H_8$]-imatinib, [$^{13}C$, $^2H_3$]-nilotinib and [$^2H_8$]-dasatinib (all >99%) were obtained from Alsachim, Illkirch, France. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formate, hexane and ethylacetate were purchased from Fluka (Buchs, Switzerland), Merck (New York, USA) and Carlo Erba (Val de Reuil, France), respectively. Formic acid (FA) (98%) and methanol for chromatography Lichrosolv® (MeOH) were obtained from Merck (Darmstadt, Germany).

6 mL BD Vacutainer® blood collection tubes with lithium heparin and EDTA were obtained from BD, New York, USA. DMS blood spot cards were supplied by Agilent (Santa Clara, US). Harris Uni-Core I.D. 8 mm puncher was purchased from Sigma–Aldrich (Stainheim, Germany). Sachets of silica gel for the storage of blood spot cards were obtained from local market.

### 2.2. Working solutions

Primary stock solutions of imatinib, nilotinib and dasatinib were prepared by dissolving an accurately weighted amount of each drug in MeOH to yield 1 mg/mL, 1 mg/mL and 0.05 mg/mL drug concentrations, respectively. Primary stocks were diluted 10-times with 0.1% FA in water to give secondary stocks, and secondary stock solutions were further diluted 10-times with 0.1% FA in water to give tertiary stocks. Working solutions (WS) were prepared by mixing appropriate volumes of certain stock solution of each compound to desired concentration of imatinib, nilotinib and dasatinib in each WS.

Stock solutions of [$^2H_8$]-imatinib, [$^{13}C$, $^2H_3$]-nilotinib and [$^2H_8$]-dasatinib were prepared in MeOH (imatinib and nilotinib) and DMSO (dasatinib) in 1 mg/mL concentration and were further diluted to 2 mg/L with 0.1 FA in water to give a secondary stock. The final internal standard (IS) concentration (0.66 mg/L of each compound) was achieved by mixing equal volumes of secondary stock of each stable isotope labelled compound.

All the solutions were stored at −20 °C and brought to room temperature before use.

### 2.3. Calibration sample preparation

Calibration standards (CS) and quality control (QC) samples were prepared by diluting working solutions either with fresh blank whole blood (haematocrit 42%) or with plasma. Heparinised blood was used in this case, because this anticoagulant does not interfere with the drug transport into the blood cells. However our previous experiments showed no significant differences in sample processing and analysis due to the use of EDTA or heparinised blood. CS and QC samples prepared in blood were equilibrated for 2 h at 37 °C then 10 μL of blood was spotted directly onto the DBS cards using a volumetric pipette. The cards were allowed to air dry for at least 2 h at room temperature prior to processing or storage. Plasma CS and QC were used immediately or stored at −20 °C.

The final concentrations of CS and QC are shown in Table 1 (available as supplementary data) and were selected to mimic the expected blood or plasma TKI concentrations in CML patients.

### 2.4. Patient sampling

The study protocol was approved by the Slovenian National Medical Ethics Committee and informed verbal and written consent was obtained from each patient. 2 mL of EDTA blood was collected from 22 CML patients on therapy with imatinib (18), dasatinib (3) or nilotinib (2) at times when other blood samples were being withdrawn for routine laboratory analysis. To prepare a DBS sample, 10 μl of EDTA blood was spotted four times, using a volumetric pipette, onto the Agilent DBS Cards and allowed to dry for at least 2 h at room temperature. All DBS cards were stored in a plastic bag

with a desiccator until analysis. At the same time the rest of the blood taken was centrifuged and 500 μL of plasma was stored and afterwards used to determine the plasma concentration of the same drugs with already validated LC–MS/MS method.

### 2.5. Dried blood spot and plasma sample extraction

For DBS extraction whole blood spot was punched out of the DBS card and transferred to a well in a 96-well Captiva ND$^{Lipids}$ (Agilent, NY, USA) filter plate, specially designed to remove phospholipids, previously precipitated proteins, surfactants and other matrix interferences from biofluids. 50 μL of internal standard solution and 300 μL of 0.1% FA in MeOH were added to each well and mixed with 5 pipette aspirations of 3/4 combined liquid volume. After vacuum application the extract was collected in Captiva 96-well collection plate and transferred to the autosampler for subsequent LC–MS/MS analysis.

Plasma samples were processed by liquid–liquid extraction. In 2 mL Eppendorf safe lock tube 200 μL of plasma was alkalised with 200 μL of 0.2 M NaOH and spiked with 50 μL of internal standard solution. Afterwards 1.000 mL of organic phase consisting of hexane:ethyl acetate (30:70, v/v) was added. The mixture was vortexed thoroughly for 1 min and further shaken on an orbital shaker for 40 min (200 rpm). After that 800 μL of organic phase was transferred to a clean Eppendorf safe lock tube and dried with $N_2$ in TurboVap (Zymark, MA, USA). The dried extract was reconstituted in 250 μL mixture of 50% 4.5 mM ammonium formate and 50% MeOH by vortexing for 1 min, transferred to a 96-well plate and analysed by LC–MS/MS.

### 2.6. Chromatographic and mass spectrometry conditions

The analysis of extracted DBS and plasma samples was performed on an Agilent 1290 Infinity UHPLC coupled to an Agilent 6460 Triple-Quadrupole mass spectrometer equipped with a JetStream$^{TM}$ ESI source (Agilent Technologies, Inc., Santa Clara, CA, USA). Chromatographic separation was performed on a Kinetex 50 mm × 2.1 mm C18 column (2.6 μm particles), guarded by a 4 mm × 2 mm C18 cartridge column (Phenomenex, Torrance, USA). The column temperature was maintained at 50 °C. The autosampler was fitted with a 20 μL injection loop. The injection volumes for DBS and plasma extracts were 1.0 and 0.1 μL, respectively. The mobile phase A was 0.1% FA in MilliQ water and mobile phase B was 100% acetonitrile. The flow rate was 0.65 mL/min and the following linear gradient was employed (% of mobile phase B): 10, 20, 30, 50, 68 and 10 at the corresponding time points: 0, 0.5, 1, 1.25, 1.6 and 1.8 min, respectively. The total run time was 2.6 min. The retention times of imatinib, nilotinib and dasatinib were 1.21, 1.61 and 1.38 min, respectively (Fig. 1).

The mass spectrometer was operated in positive ESI mode. The drying gas temperature and the flow rate were 275 °C and 5 L/min, respectively, and the sheath gas temperature and flow rate were 320 °C and 11 L/min, respectively. The nebuliser pressure was 45 psi (0.31 MPa) and the capillary voltage was 4000 V. The mass spectrometer was operated in MRM mode (Table 1). Both quadrupoles were set to 2.5 units mass resolution, respectively, and the dwell times were 50 ms for each $m/z$ channel. Instrument control, data acquisition and quantification were performed by MassHunter Workstation software B.03.01 (Agilent Technologies, Torrance, USA).

## 3. Method validation

Method validation was performed based on the recommendations published by the Food and Drug Administration (FDA) [27] and following the recommendations and guidelines for DBS methods use in regulated bioanalysis by Houghton [28]. Absolute and relative matrix effect was estimated according to Matuszewski et al. [29].

### 3.1. Selectivity

The selectivity of the developed method was determined by analysing blank DBS samples from six individuals, prepared according to proposed extraction procedures. Blank sample chromatograms were compared with those at the lower limit of quantification (LLOQ). Thus it was shown that no interfering peaks were present in the biological matrix at the retention times of either compound.

### 3.2. Linearity and lower limit of quantification

Quantitative analysis of the TKIs in DBS and plasma samples was performed using the IS method. Weighted linear regression model for each compound was used to calculate the equation relating a peak area ratio of analyte vs. IS and nominal analyte concentration in each calibration standard. To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited.

The lower limit of quantification (LLOQ) was defined by the concentration, where inaccuracy and imprecision did not exceed 20%.

### 3.3. Accuracy and precision

The inter- and intra-day accuracy and precision of the developed assay were determined with three QC DBS samples, containing all three substances in concentrations 300, 800 and 2000 μg/L for imatinib and nilotinib and 15, 40, 100 μg/L for dasatinib (QC1, QC3 and QC5). The three concentrations were chosen to encompass the whole range of the calibration curve corresponding to the drug levels expected to occur in most patient samples. Within-day accuracy and precision were calculated on a single day using five replicates at each concentration. Between-day accuracy and precision were calculated using five replicates at each concentration over three consecutive days. Calibration standards were prepared on the day of analysis. The accuracy was expressed as the relative error (RE) and precision as the coefficient of variation (CV). A RE and CV of ≤15% at all concentrations were considered acceptable, regarding the FDA guidelines [27].

During the routine analysis of patient samples, the same QC samples were analysed in triplicate at the beginning and at the end of the run. The analytical series were considered valid and accurate only if the percentage deviation between theoretical and back-calculated (experimental) concentrations for each quality control sample were less than ±15%.

### 3.4. Matrix effects and recovery

The influence of matrix on electrospray ionisation and assay recovery were evaluated by analysing three differently prepared QC samples in three replicates: (A) DBS QC sample extracts; (B) blank DBS QC sample extracts post-spiked with both the analyte and the IS concentrations equivalent to A; and (C) neat solution QC samples prepared in mobile phase in concentrations of the analytes and the IS equivalent to A and B. The matrix effect was calculated as peak area ratio of $B/C \times 100\%$; if the ratio was between 85% and 115%, an absolute exogenous matrix effect was excluded.

Recovery and overall process efficiency were calculated by comparing the responses from pre-extraction spiked samples with

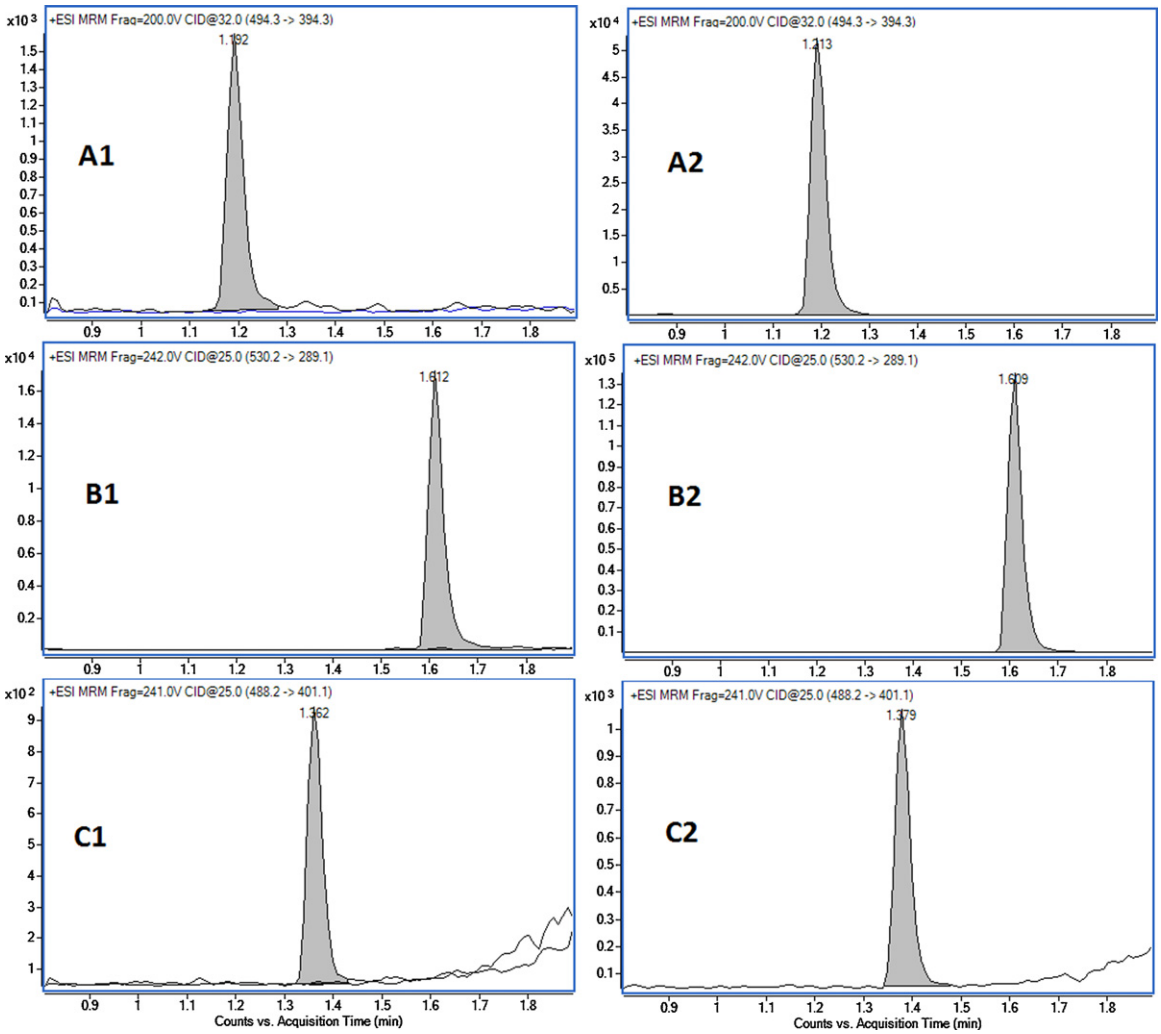


**Fig. 1.** MS chromatograms on the left hand side are recorded from a DBS sample at LLOQ (A1, B1 and C1) showing good signal to noise ratio for imatinib, nilotinib and dasatinib, respectively. An extracted DBS blank chromatogram is overlaid with A1, B1 and C1 and shows excellent selectivity without any matrix interferences. Typical DBS sample chromatograms obtained from patients taking either imatinib, nilotinib or dasatinib are presented on the right hand side (A2, B2 and C2 respectively).

those from post-extraction spiked samples ($A/B \times 100\%$) or neat solutions ($A/C \times 100\%$), respectively.

Even more important than the low absolute matrix effect is the absence of the relative matrix effect, which assures that the method accuracy in not compromised by matrices originating from different individuals. According to Matuszewski [29] five DBS calibrator standards (300, 600, 800, 1000 and 2000 μg/L) were prepared in five different lots of blood and standard curves were constructed. Slopes of the standard lines were determined from the linear regression analysis of the peak area ratios of drug/IS versus analyte concentrations. The variance in slopes was used to evaluate the absence or presence of a relative matrix effect (resulting from the combination of the effect of matrix on both recovery of analytes from different lots and ion suppression or enhancement between different lots). If the regression slope's RSD was below 3–4%, the method was considered free from significant relative matrix effect.

**Table 1**
The MRM transitions and fragmentation parameters used for the quantification of TKIs using their isotopically labelled analogues as internal standards.

| Analyte | MRM $m/z$ transition | Fragmentor (V) | Collision energy (eV) |
|---|---|---|---|
| Imatinib | 494.3 → 394.3 | 200 | 32 |
| $D_8$-imatinib | 502.3 → 394.3 | 200 | 32 |
| Nilotinib | 530.2 → 289.1 | 242 | 25 |
| $^{13}C$-$D_3$-nilotinib | 534.2 → 289.1 | 244 | 25 |
| Dasatinib | 488.2 → 401.1 | 241 | 25 |
| $D_8$-dasatinib | 496.2 → 406.1 | 244 | 25 |

### 3.5. Haematocrit

To estimate the effect of haematocrit (Hct) on the accuracy of analyte quantification, QC samples were prepared at concentrations of 300, 800 and 2000 μg/L, using blood with various Hct values (0.30, 0.40 and 0.60). Blood with various erythrocyte percentages was prepared by diluting concentrated human erythrocytes with fresh human plasma. Measured concentrations were compared and RSD (%) was calculated.

### 3.6. Stability

We investigated the effect of storage time and storage temperature on the stability of spiked DBS samples. To assess that, quality control DBS samples were analysed after 7, 14 and 28 days storage at room temperature, and after 3 days storage at 40 °C and −20 °C. Measured concentrations of stored samples were compared to those obtained after analysis of freshly spiked DBS samples. RSD of less than 15% was considered acceptable.

**Table 2**
Linear response range, mean slope and intercept values and Pearson's coefficient of correlation ($R^2$) for calibration curves of imatinib, nilotinib and dasatinib.

| TKI | Linear response range (μg/L) | Intercept | Slope | $R^2$ |
|---|---|---|---|---|
| Imatinib | 50–5000 | −0.00571 | 0.00039 | 0.999 ± 0.002 |
| Nilotinib | 50–5000 | 0.01802 | 0.00260 | 0.998 ± 0.001 |
| Dasatinib | 2.5–250 | −0.00509 | 0.00472 | 0.997 ± 0.002 |

### 3.7. Patient sample analysis

Developed and validated method was clinically applied on 23 DBS patient samples. Plasma concentrations calculated from DBS values after haematocrit (Hct) correction: $C_{plasma} = C_{DBS}/(1 - Hct)$ were plotted against plasma concentrations determined after liquid–liquid extraction using linear regression. To confirm that the DBS method gives comparable results to those obtained by plasma concentration determination a Bland–Altman approach was used (MedCalc software, Belgium).

## 4. Results

### 4.1. Selectivity

Fig. 1 shows three out of six blank chromatograms recorded and overlaid with imatinib, nilotinib and dasatinib LLOQ sample chromatograms. No significant interferences at the retention times of either compound were detected.

### 4.2. Linearity

A weighted 1/concentration (1/*X*) linear regression was used to construct the calibration curve by plotting analyte/IS ratio vs. nominal analyte concentration. The back calculated concentrations for each calibrator standard expressed as a relative error were between 0.2% and 14.2% for imatinib, between 0.7% and 13.0% for nilotinib and between 2.3% and 11.1% for dasatinib over the calibration range. Linear response was observed over a wide concentration range. The mean slope and intercept values for the calibration curves are given in Table 2.

### 4.3. Accuracy and precision

In order to assess the robustness of the method, inter and intra-day accuracy and precision were evaluated. Data are given in Table 3. All the values obtained were within the guidelines published by the FDA [27]. The inter-day precision for the QC1, QC3 and QC5 were between 7.1% and 10.3% for all the compounds and the accuracy was within ±15%. Therefore, the method presented acceptable accuracy and precision.

### 4.4. Matrix effect and recovery

Matrix effect occurs when a biological sample contains a component that does not give a signal in the MRM channel used for the quantitation of an analyte but co-elutes with the analyte and affects (attenuates or enhances) the response of the analyte. The assessment of the matrix effect constitutes an important and integral part of validation for a quantitative LC–MS/MS method, since the presence of a matrix effect can dramatically affect sensitivity, accuracy and/or precision of a bio-analytical method. Normally, the accuracy/precision problem can be solved by using a co-eluting stable isotope analogue as an IS, like [$^2H_8$]-imatinib, [$^{13}C$, $^2H_3$]-nilotinib and [$^2H_8$]-dasatinib used with this method.

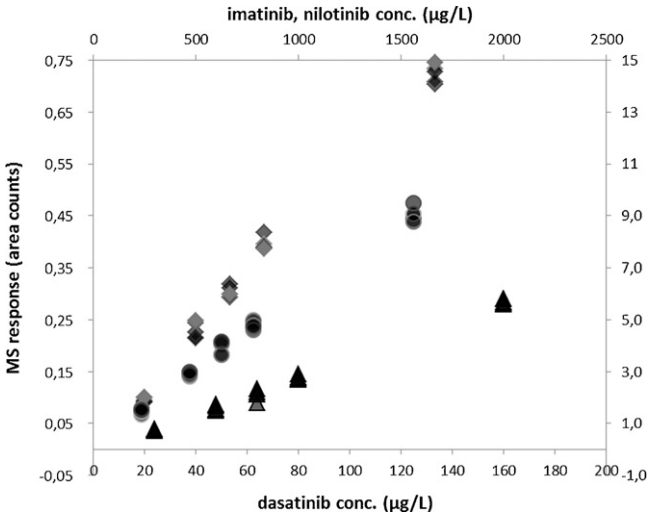


**Fig. 2.** Analysis of QC samples of imatinib (♦), dasatinib (●) and nilotinib (▲) prepared in different blood lots ($n = 5$).

Table 4 shows that based on the quantitative assessment, the estimated matrix effect (% ME) on imatinib, dasatinib and nilotinib was found to be within 94.5% and 106.7%, which indicates that method is free from any major ion suppression or enhancement for all three substances. Overall mean recovery at the three quality control sample levels were consistent and reproducible for all three TKIs. The values were close to 100% and variability (% CV) at each concentration was found to be less than 15%. The method displayed good overall process efficiency.

Relative matrix effect was estimated by comparing slopes of the calibration curves, constructed from measured QC samples (QC1–QC5) prepared in different blood lots and plotted against the nominal concentration of each analyte in DBS QC sample (Fig. 2). Coefficient of variability between slopes was 1.5–2.8% for imatinib, 0.8–3.4% for dasatinib and 1.0–3.9% for nilotinib. No significant difference in slope values indicates a lack of relative matrix effect.

### 4.5. Haematocrit

Haematocrit levels are known to vary between individuals and with disease state, and since TKIs also distribute into red blood cells, haematocrit effect on the accurate quantification of analytes within DBS samples was examined. RSD was calculated for three QC samples (QC1, QC3 and QC5) prepared in blood with various Hct values. No significant impact of Hct on accuracy was observed with either imatinib (2.0–14.0%), dasatinib (1.7–13.3%) or nilotinib (1.6–13.0%).

### 4.6. Stability

One of the advantages of DBS sampling is increased stability of the analyte in these samples, allowing collection, storage and delivery at room temperature. This was proven also with our substances. All three TKIs in DBS samples proved to be stable at room temperature, no matter whether they were stored for 7, 14 or 30 days. The same applies for storage at −20 °C. However, storage at 40 °C obviously caused a significant degradation of dasatinib, which concentration fell to almost 60% of the one measured in a freshly prepared DBS sample (Table 5). Based on these results storage at room temperature or lower is recommended.

**Table 3**
Inter and intra-day accuracy and precision.

| | Imatinib | | | Dasatinib | | | Nilotinib | | |
|---|---|---|---|---|---|---|---|---|---|
| Nominal conc. (μg/L) | 300 | 800 | 2000 | 15 | 40 | 100 | 300 | 800 | 2000 |
| Intra day statistics | | | | | | | | | |
| Mean conc. (μg/L) | 315 | 769 | 2001 | 13.1 | 42.3 | 99.1 | 309 | 831 | 2008 |
| Accuracy (% bias) | 4.9 | −3.9 | 0.1 | −12.9 | 5.7 | −0.9 | 3.1 | 3.9 | 0.4 |
| Precision (% CV) | 3.1 | 4.4 | 5.6 | 2.8 | 4.9 | 7.1 | 6.9 | 5.3 | 6.8 |
| Inter day statistics | | | | | | | | | |
| Mean conc. (μg/L) | 312 | 726 | 1994 | 13.0 | 41.4 | 99.6 | 324 | 783 | 1915 |
| Accuracy (% bias) | 4.1 | −9.3 | −0.3 | −13.2 | 3.4 | −0.4 | 7.8 | −2.2 | −4.3 |
| Precision (% CV) | 9.1 | 8.1 | 8.2 | 9.9 | 9.3 | 8.2 | 10.3 | 7.7 | 7.1 |

**Table 4**
Absolute matrix effect, recovery and overall process efficiency for DBS analysis of imatinib, dasatinib and nilotinib in blood samples.

| | Imatinib | | | Dasatinib | | | Nilotinib | | |
|---|---|---|---|---|---|---|---|---|---|
| Nominal conc. (μg/L) | 300 | 800 | 2000 | 15 | 40 | 100 | 300 | 800 | 2000 |
| Matrix effect (%) | 106.0 | 106.7 | 99.6 | 102.9 | 102.4 | 98.4 | 94.5 | 95.8 | 103.1 |
| Recovery (%) | 97.4 | 94.8 | 94.5 | 96.1 | 93.5 | 90.5 | 108.4 | 103.5 | 93.1 |
| Process efficiency (%) | 103.2 | 101.1 | 94.1 | 98.9 | 95.8 | 89.0 | 102.4 | 99.3 | 97.1 |

**Table 5**
Influence of storage time and temperature on imatinib, dasatinib and nilotinib stability in DBS samples. Results present a percentage of drug determined relative to those obtained after the analysis of freshly prepared DBS samples.

| Storage conditions | Imatinib | Dasatinib | Nilotinib |
|---|---|---|---|
| 7 days at 25 °C | 94.8–110.5 | 90.9–103.4 | 89.3–94.9 |
| 14 days at 25 °C | 96.3–107.5 | 85.8–101 | 95.5–104.4 |
| 28 days at 25 °C | 86.6–103.4 | 86.0–92.9 | 85.9–90.8 |
| 3 days at −20 °C | 93.8–107.1 | 86.8–102.5 | 88.5–96.8 |
| 3 days at 40 °C | 88.7–101.4 | 63.7–83.2 | 87.4–94.6 |

## 5. Clinical application

The selective, sensitive, accurate and precise method developed was used to analyse 24 CML patient DBS samples on therapy with imatinib (18), dasatinib (3) or nilotinib (2). The concentration range for all DBS samples analysed was found to be between 604 and 1676 μg/L for imatinib, 3.6–98.7 μg/L for dasatinib and 586–860 μg/L for nilotinib which is above the LLOQ (50 μg/L for imatinib and nilotinib/2.5 μg/L for dasatinib) and below the highest calibration point (5000 ng/mL for imatinib and nilotinib/250 μg/L for dasatinib) of the method. Fig. 1 (right hand side) shows typical chromatograms of three patient DBS samples containing imatinib, nilotinib and dasatinib. Values obtained from analysis of DBS samples were compared to the plasma concentrations determined by our previously validated LC–MS/MS method. Fig. 3 shows the relationship between imatinib, nilotinib and dasatinib levels in plasma and corresponding dried blood spots. A good correlation ($R^2$ = 0.9772) was observed between both measurements after the haematocrit correction. A slope value slightly higher than 1 (1.102) indicates higher concentration values determined from blood samples, which probably originate from the cell membrane associated drug and from partial extraction of TKIs from red blood cells also determined in case of blood analysis [30,31]. However, because blood to plasma partitioning coefficients are 1.42 for imatinib [31], 1.3 for nilotinib [32] and 1.8 for dasatinib [33] and thus lower than 2 [36], no significant errors in the concentrations determined due to minor erythrocyte lysis is expected. Our experiment with different Hct values also showed that both the accuracy and the precision of our method remained uncompromised over a wide Hct range.

The difference between DBS and plasma sample measurements was further analysed using the Bland–Altman difference plot (Fig. 4). Because of different concentration ranges for imatinib, nilotinib and dasatinib, the % difference between the methods was plotted against the averages of the values obtained from the two techniques. Despite the mean −7.8% difference between the methods, which is probably a consequence of the above-mentioned reasons, all the points lie between the limits of agreement (mean ± 1.96 SD). This indicates that the two methods can be used interchangeably when monitoring patient's TKI levels.

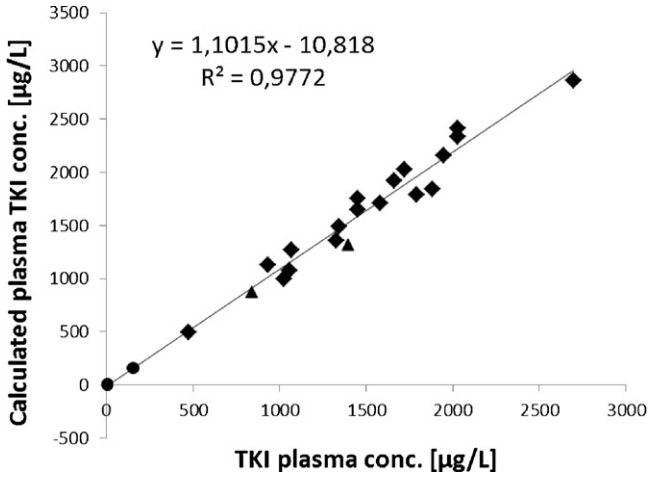


**Fig. 3.** The relationship between imatinib (♦), nilotinib (▲) and dasatinib (●) levels in plasma determined by previously validated LC–MS/MS method (*x* axis) and plasma concentrations calculated from corresponding dried blood spot concentrations and haematocrit (Hct) values (*y* axis).

However, another issue arises regarding clinical application of the above-described method. Because true DBS samples are usually taken by finger prick, that means that capillary blood is used instead of venous as described in our paper. The use of different sampling sites (venous vs. capillary) can in some cases also give rise to different drug concentration measurements [34]. This difference is often present with low molecular size, high lipid solubility and relatively low protein binding drugs (TKIs are not examples of such drugs) and even then only in the absorption phase of the drug. After distribution equilibrium is attained – this applies to our clinical samples as well – the difference between venous and capillary blood should not be significant [35]. Based on all the above-described results, we can safely assume that our method presents a reliable alternative to other methods used in TDM of TKIs.

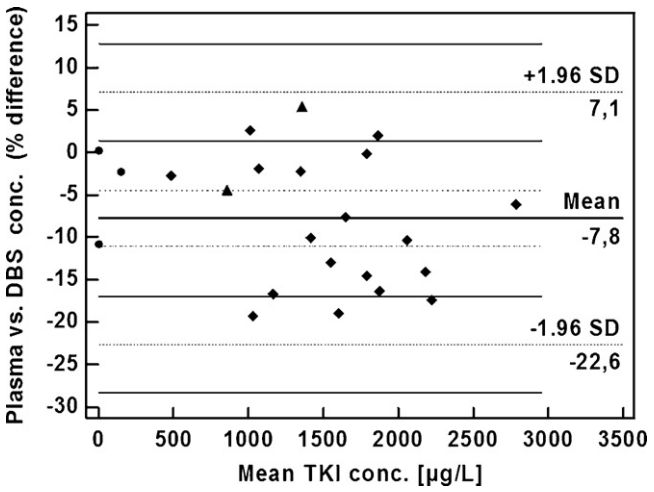


**Fig. 4.** Bland–Altman relation showing the % difference in imatinib (♦), nilotinib (▲) and dasatinib (●) concentrations obtained from plasma samples and from dried blood spot (DBS) analysis after haematocrit correction. The central horizontal dotted lines represents the mean difference or bias. The two other dotted lines (expected +2SD and expected −2SD) represent the expected distribution of 95% of the measured points, as determined by the combined total variation of each individual method.

## 6. Conclusion

Blood level testing is often performed in the therapy of patients treated with imatinb, so many different methods have been developed to process these samples. Present paper describes another alternative – simultaneous determination of three TKIs currently used in therapy of CML from DBS samples using a LC–MS/MS method. Dried blood spot sampling has gained increasing popularity in last years. Due to its numerous practical advantages, like the ease of sample collection, storage and delivery, it has become a good alternative to traditional plasma sampling. Our method proved to be rapid, sensitive, selective, accurate and precise and gave reliable and reproducible results also whilst analysing patient samples. A good correlation between drug levels measured in plasma and in the corresponding DBS was observed. Due to its ease of performance and convenient sample collection, it can be considered very useful for TDM of these drugs.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.07.011.

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