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Automated high throughput analysis of antiretroviral drugs in dried blood spots

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For therapeutic drug monitoring in remote settings, dried blood spots (DBS) are particularly advantageous, as blood sample collection and handling is uncomplicated. The aim of this study was to develop and validate an automated extraction method for the analysis of nevirapine, efavirenz and lopinavir in DBS samples. Automated extraction was performed with methanol : water (70 : 30 *v/v*), using a DBS-MS 500 autosampler coupled to a liquid chromatography tandem mass spectrometry system. The autosampler used digital images of each DBS to position the extraction head, sprayed 10 μl of internal standard onto each DBS and extracted a 4-mm disc (Ø) from the centre of each spot by unilateral flow using 25-μl extraction solvent. The analytes were baseline separated on a pentafluorophenyl column and analysed by using electrospray ionization with multiple reaction monitoring in positive polarity mode for nevirapine and lopinavir and in negative mode for efavirenz. The method was linear between 10 and 10 000 ng/ml for all analytes. Automated sample extraction resulted in consistent recoveries (nevirapine: 70 ± 6%, efavirenz: 63 ± 11% and lopinavir: 60 ± 10%) and matrix effects between different donors and concentration levels. Intra-day and inter-day accuracy and precision deviations were ≤15%. Manual and automated extractions of DBS samples collected within the framework of an adherence assessment study in rural Tanzania showed good agreements with deviations of less than 10%. Our study highlights that therapeutic drug monitoring samples obtained in the resource-constrained setting of rural Africa can be reliably determined by automated extraction of DBS. Overall, automatization improved method sensitivity and facilitates analysis of large sample numbers. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: nevirapine; efavirenz; lopinavir; liquid chromatography tandem mass spectrometry; dried blood spots; automated extraction

Introduction

The dried blood spot (DBS) technique facilitates minimally invasive blood sampling, whereby capillary blood is spotted onto a filter paper, ideally leading to a homogenous blood spot. After complete drying, a fixed blood spot area can be punched out for drug analysis, making exact pipetting unnecessary.[1] In contrast to conventional plasma sampling, the DBS technique does not require a trained phlebotomist. Only a few drops of blood are withdrawn after a simple and minimally invasive finger prick: a process that can even be performed by adequately instructed patients.[2] Moreover, DBS samples minimize biohazard risk during further sample handling and are generally stable at room temperature.[3] Hence, the collection of DBS samples is especially attractive for field studies in remote or resource-constrained settings, where uninterrupted cold chains cannot be guaranteed.[4]

Worldwide, an estimated 37 million people are HIV positive, of which the majority reside in Sub-Saharan Africa.[5] Antiretroviral therapy leading to viral suppression has been strongly correlated with increase in survival and improved quality of life.[6,7] Good adherence to antiretroviral therapy is paramount, and patients with suboptimal adherence are at risk of HIV progression and the development of drug resistance, which consequently narrows options for future treatment.[6,8] Circulating antiretroviral drug concentrations are characterized by a high degree of between-patient variability, due to genetic and nongenetic heterogeneity in drug disposition. Therapeutic drug monitoring (TDM) is an approach to standardize drug exposure through dosage individualization and to prevent both the toxicity associated with high exposure and the inefficacy associated with insufficient exposure.[9–12] However, particularly in resource-limited settings,

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TDM is infrequently performed due to technical challenges.[6,8,13] In such settings, the previously mentioned advantages of DBS sampling could facilitate implementation of regular TDM.

On the other hand, the development of bioanalytical methods for DBS samples is more complex than for conventional liquid matrices, as variable haematocrit values alter not only analyte recovery but also the extent of blood diffusion within the filter paper, thereby impacting the reliability of the DBS analysis.[14] Moreover, the small amount of blood collected on a filter card requires highly sensitive bioanalytical methods.[14] Finally, concentrations in DBS samples must be compared with conventional plasma samples to allow correct interpretation of DBS measurements.[15–18]

The aim of the present work was to develop and validate a fully automated DBS extraction method for the analysis of nevirapine, efavirenz and lopinavir, with sufficient robustness to handle large sample numbers. Certain steps of the validation procedure such as assessment of extraction recovery and matrix effects are more complex when automated extraction systems are used and require modifications of standard validation procedures. Feasibility of the automated extraction method was demonstrated by comparing concentrations obtained after automated and manual DBS extraction of samples from a combination antiretroviral therapy adherence assessment study conducted at the Chronic Diseases Clinic of Ifakara, Tanzania. The DBS samples of patients treated with the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine and the protease inhibitor lopinavir were collected.[19] In contrast to previous studies that used manual DBS extraction for the measurement and validation of combination antiretroviral therapy compounds,[20–23] we extracted DBS by using a fully automated DBS autosampler (CAMAG, DBS-MS 500).[24] This autosampler exhibits a TLC-based extraction head, with a circular plunger that seals a vent of 4-mm inner diameter on the blood spot. The extraction solvent passes horizontally from the inlet capillary through the blood spot to the outlet capillary and into a sample loop (unilateral extraction). Thus, in contrast to other online DBS extraction systems,[25–28] the extraction solvent is not forced vertically through the filter paper (flow-through extraction). The autosampler is connected to a liquid chromatography tandem mass spectrometry (LC–MS/MS) system, features 500 DBS card slots, takes an image of the blood spot before and after the extraction process, sprays the internal standard solution onto each blood spot and works with a low volume (~25 μl) of extraction solvent.

Materials and methods

Chemicals, reagents, and reference compounds

Gradient grade water and methanol for liquid chromatography as well as formic acid (98–100%) were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, USA). The reference compounds, nevirapine, efavirenz and lopinavir and its deuterated internal standards (IS) nevirapine-d3, efavirenz-d5 and lopinavir-d8 were products of Toronto Research Chemicals (Toronto, Canada). DBS cards (grade 226 filter paper) were kindly provided by CAMAG (Muttenz, Switzerland). Fresh whole blood was obtained from the local blood donation centre (Basel, Switzerland).

LC–MS/MS instrumentation and settings

Chromatography was performed on a modular high-performance liquid chromatography system from Shimadzu (Kyoto, Japan); it contained a system controller (CBM-20A), four pumps (2× LC-20AD and 2× LC-20AD XR), a degasser (DGU-20A5) and a column oven (CTO-20A). A CTC HTS PAL autosampler (CTC analytics, Zwingen, Switzerland) was used in the case of manual extraction of DBS samples. Automated extractions were carried out with a DBS-MS 500 autosampler (CAMAG, Muttenz, Switzerland). Analytes were separated on a Kinetex 2.6 μ F5 100 Å (50 × 2.1 mm) analytical column (Phenomenex, Torrance, USA). A filter frit (SS 0.5 μm 0.62 × 0.65, Ercatech AG, Bern, Switzerland) was connected upstream to the analytical column. Mobile phase A consisted of water plus 0.1% formic acid, while methanol supplemented with 0.1% formic acid was used as mobile phase B. The following stepwise gradient was applied: 5% (0–0.25 min), 5%–60% (0.25–0.4 min), 60%–80% (0.4–2.0 min), 80%–95% (2.0–2.2 min), 95% (2.2–3.0 min) and 5% (3.0–3.3 min). The flow rate was set at 0.5 ml/min at 45 °C. The high-performance liquid chromatography liquid stream was connected to an API 4000 Q-trap tandem mass spectrometer (AB Sciex, Framingham, MA, USA) only between minute 0.6 and 2.5 of each run to reduce system contamination. The analytical run was divided into three multiple reaction monitoring periods, whereas electrospray ionization was switched from positive to negative mode between minute 1.3 and 1.7 (period 2) of each run. The following mass transitions and compound specific settings were used: 267 → 226 *m/z* for nevirapine [declustering potential (DP): 76 V, collision energy (CE): 20 V, entrance potential (EP): 10 V, collision cell exit potential (CXP): 16 V], 270 → 229 *m/z* for nevirapine-d3 (DP): 121 V, CE: 37 V, EP: 10 V, CXP: 16 V), 314 → 244 *m/z* for efavirenz (DP: −95 V, CE: −26 V, EP: −10 V, CXP: −13 V), 319 → 248 *m/z* for efavirenz-d5 (DP: −75 V, CE: −28 V, EP: −1 V, CXP: −15 V), 629 → 155 *m/z* for lopinavir (DP: 111 V, CE: 35 V, EP: 10 V, CXP: 10 V) and 637 → 163 *m/z* for lopinavir-d8 (DP: 66 V, CE: 75 V, EP: 10 V, CXP: 10 V). The general settings of the mass spectrometer were as follows: ion source gas-1 60 l/min (N_2), ion source gas-2 50 l/min (N_2), curtain gas 10 l/min, collision gas 4 l/min, ion spray voltage 5500 V (positive mode) and −4200 V (negative mode) and source temperature 350 °C. ANALYST software 1.6.2 (AB Sciex, Framingham, MA, USA) was used to operate the LC–MS/MS system.

Preparation of standards and extraction solvents

Nevirapine, efavirenz and lopinavir stock solutions were prepared in DMSO (10 mg/ml) and stored at −20 °C. Stock solutions were pooled and serially diluted with DMSO to cover a range from 1000 to 1 μg/ml. The dilution series for calibrators and QC samples originated from different weightings. IS stock solutions were likewise prepared in DMSO at a final concentration of 1 mg/ml. The extraction solvent was a mixture of methanol and water (70 : 30 *v/v*). The IS was dissolved in methanol at a concentration of 1 μg/ml for nevirapine-d3 and at 2 μg/ml for efavirenz-d5 and lopinavir-d8.

Preparation of calibration and quality control samples

Freshly collected human blood was obtained from the local blood donation centre (Basel, Switzerland). Ethylenediaminetetraacetic acid was used as an anticoagulation agent (vacutainer tubes, BD, Allschwil, Switzerland). Calibrators and Quality control (QC) samples were prepared by spiking blank blood with the analyte dilution series in a ratio of 1 : 100 (*v/v*). Calibration samples for automated extractions encompassed a range from 10 to 10 000 ng/ml and 50 to 10 000 ng/m for manual extractions. QC samples were prepared at the lower limit of quantification (LLOQ, 10 ng/ml), as well as at

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low (50 ng/ml), medium (500 ng/ml) and high (5000 ng/ml) concentration levels. In the case of manual extractions, the LLOQ was set to 50 ng/ml. Spiked blood samples were gently mixed and agitated on a roll-agitator (CAT RM 5 Staufen, Switzerland), after which 15-μL aliquots were spotted onto CAMAG DBS cards (CAMAG, Muttenz, Switzerland). DBS cards were dried at room temperature for at least 2 h and were subsequently stored at −20 °C in sealed plastic bags containing desiccants. Each calibration set consisted of 1 blank sample (DBS sample processed without IS), 1 zero sample (DBS sample processed with IS) and 11 calibrators (9 calibrators in the case of manual extractions). Calibration lines were established by linear regression of the nominal analyte concentration against the analyte : IS peak area ratio by using a weighting factor of $1/x^2$.

Dried blood spot sample extraction

Automated extraction

The extraction head was cleaned in an ultra sound bath at 40 °C for 10 min prior each set of analyses because filter paper debris can clog the port after several extractions. Moreover, the internal standard module was primed for at least 3 cycles to clear air bubbles from the syringe. Each extraction solvent was primed for more than 3 cycles, and the rinsing solvents were flushed for 4 min. Twisted DBS cards were pressed overnight under a heavy weight before use in the autosampler.

The DBS cards were photographed with the built-in camera of the DBS-MS 500 autosampler before and after each run to check for the presence of a blood spot and to adjust the extraction head to the centre of each spot. The software of the autosampler automatically recognized inadequate DBS based on their roundness, diameter and area. Inadequate DBS were excluded from analysis. Ten μl of internal standard was sprayed in a homogenous layer onto each spot. After a 20-s drying time, the samples were extracted with a volume of 25 μl and a 40-μl/min flow rate. As a 20-μl loop was installed, the first 5 μl of each extraction was discarded. To complete the automated DBS extraction cycle, the system was first rinsed for 20 s with a methanol : acetonitrile : isopropanol : water (1 : 1 : 1 : 1 *v/v*) mixture, after which it was cleaned for a further 20 s with water containing 0.1% formic acid.

Manual extraction

Ten μl of internal standard was sprayed by using the internal spraying device of the DBS-MS 500 autosampler onto each spot. The card was left to dry at room temperature, and then a disc of 3 mm in diameter was manually punched out from the centre of each spot by using a manual hole puncher (Whatman, Sanford, ME, USA) and transferred to a 0.75-ml autosampler matrix tube (Thermo Scientific, Reinach, Switzerland). Afterwards, 200-μl extraction solvent, methanol : water (70 : 30 *v/v*), was added to each disc. The samples were mixed for 3 min, centrifuged (30 min; 3220 g; 10 °C, Eppendorf, Hamburg, Germany) and kept at 10 °C in the autosampler. To perform the analysis, an aliquot of 20-μl supernatant was injected into the LC–MS/MS system. Subsequently, the system was washed with methanol and a methanol : water mixture (1 : 1 *v/v*).

Method validation

The automated DBS extraction LC–MS/MS method was validated following the FDA guidance for bioanalytical method validation for industry.[29] The method was validated in terms of selectivity, sensitivity, accuracy, precision, linearity, extraction recovery, matrix effect and analyte stability. In addition, the impact of the applied blood volume was evaluated during the validation process.

Selectivity and sensitivity

Blank DBS samples from seven different subjects were examined for interfering endogenous matrix components. The signal at the designated LLOQ was set to be at least five times higher than the noise signal, with a bias in precision of less than 20% and accuracy between 80 and 120%.

Linearity

The coefficients of variation (R^2) of the linear regression, between the analyte peak area, normalized by the internal standard peak area and the nominal concentration, had to be ≥0.99. At least 75% of the calibration samples had to be within ±15% (LLOQ: ±20%) of the nominal value.

Intra-day and inter-day accuracy and precision experiments

The accuracy and precision of the method were determined by analysing QC samples from seven different subjects at four concentration levels (LLOQ, low-level, medium-level and high-level QC). Placing the QC samples between two calibration lines, one spot was analysed per condition (n = 28 QC samples, n = 20 calibrators). Precision and accuracy were evaluated within a single validation run (intra-day) as well as between three runs recorded on different days (inter-day). The precision was calculated as the percentage relative standard deviation (CV, %) for each QC concentration within an analytical run (intra-day precision, n = 7) and over all three runs (inter-day precision, n = 21). A precision of <15% (<20% at the LLOQ) was accepted in our study. The accuracy was assessed from the overall mean of each QC concentration divided by its nominal value (bias, %). A mean accuracy of 85–115% (LLOQ: 80–120%) was acceptable; however, at least 67% of the QC samples of each concentration level had to be within the acceptance range.

In addition, DBS spots using 15 and 30-μl blood were prepared at LLOQ, low, medium and high concentration levels. The change in concentration of 15 to 30 μl spots was calculated. A deviation of ≤15% (LLOQ ≤20%) implied that the method does not depend on the applied volume of blood.

Recovery and matrix effect

The extraction recovery of the DBS-MS 500 autosampler was investigated for DBS samples of seven different subjects. DBS spots at 50, 500 and 5000 ng/ml were prepared for the recovery experiments. Each spot was extracted six times for medium and high concentration samples (500 and 5000 ng/ml), while low concentration samples (50 ng/ml) were extracted three times. Between two extractions, a drying time of approximately 15 min was programmed. Using the built-in camera of the autosampler, the extraction head automatically locked onto the same area in the centre of the blood spot. The recovery was finally estimated as the percentage ratio of the analyte peak area of the first extraction to the sum of the peak areas of all subsequently conducted extractions.

Blank DBS samples from seven different subjects were prepared to quantify the effect of the blood matrix on the analyte signal intensity. The extraction solvent was spiked with 10, 100 or 1000-ng/ml nevirapine, efavirenz and lopinavir. Each blank DBS sample

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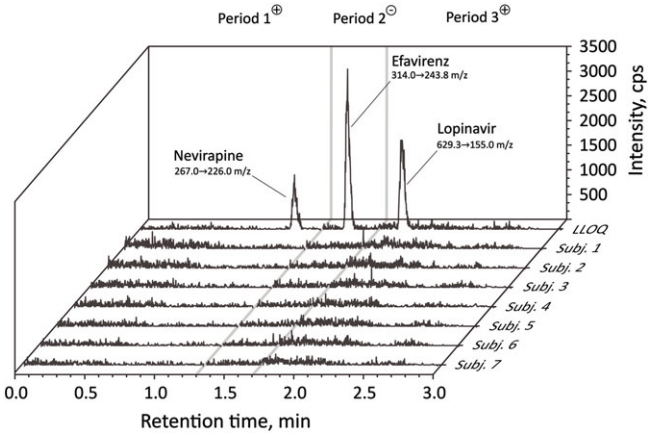

Figure 1. Chromatograms of blank DBS samples (n = 7 donors) were placed next to an LLOQ (10 ng/ml) sample. The method is selective for the quantification of nevirapine, efavirenz and lopinavir in DBS samples. [Colour figure can be viewed at wileyonlinelibrary.com]

and a corresponding card without a blood spot were processed at each concentration level. The matrix effect was calculated as the ratio of the analyte peak areas measured, following extraction of filter cards containing a blank DBS, to the peak areas of filter cards without matrix.

Stability

Stability tests of nevirapine, efavirenz and lopinavir were performed under different conditions at a medium concentration level of 500 ng/ml. Stability was evaluated in the fridge (4 °C) and the freezer (−20 °C) after 4 weeks of storage. Five replicates were analysed per condition and compared with a set of QC samples, prepared on the day of analysis.

Method application

Clinical application of the LC–MS/MS method was demonstrated by analysing a series of randomly selected DBS samples. The DBS samples contained nevirapine, efavirenz and lopinavir and were collected during an adherence assessment study conducted in Tanzania.[19] Ethical approval was obtained from the Institutional Review Board of the Ifakara Health Institute (reference no IHI 28-2013), the Tanzanian National Institute of Medical Research, Dar es Salaam, Tanzania (reference no NIMR/HQ/R.8a/V01. IX/I762) and the Tanzanian Commission for Science and Technology (no 2014-276-NA-2014-195). For each analyte, 30 DBS samples were

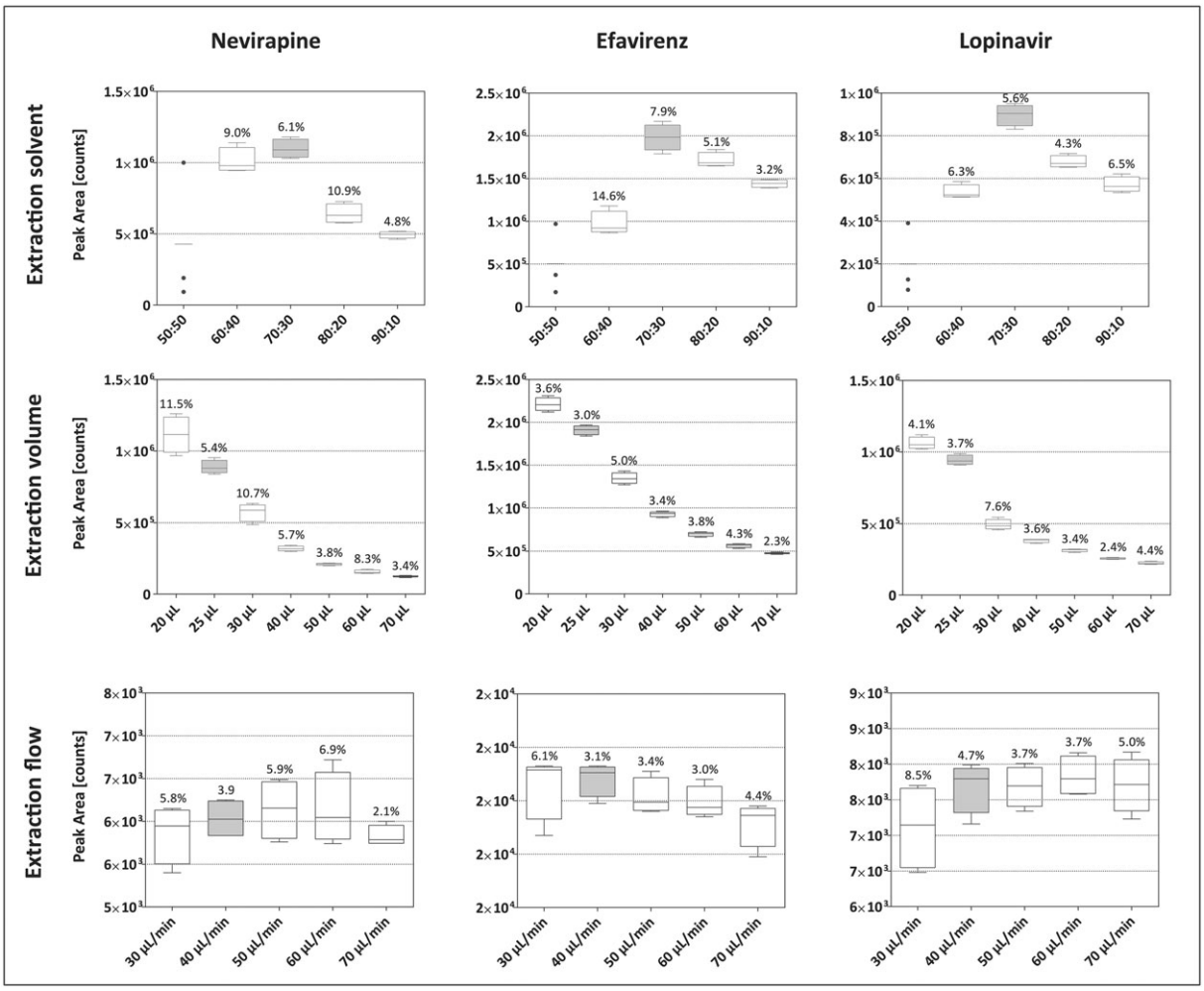

Figure 2. Optimization of the extraction parameters for the automated analysis of nevirapine, efavirenz and lopinavir from DBS samples. High concentration QC samples (n = 4) were extracted for each condition. Effect of the methanol : water mixture, extraction volume and extraction flow on the extraction yield was evaluated. Conditions with grey-coloured box plots were selected for the final extraction method. The percent figures above the boxes are CV%.

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processed by automated and manual extraction. In both cases, the IS was sprayed onto the DBS. Bland–Altman plots were created with GRAPHPAD PRISM 6.04 (La Jolla, CA, USA) to compare the two extraction methods.[30] Mean-difference plots were generated by using the mean %difference bias [%difference = (concentration automated extraction − concentration manual extraction/mean concentration) • 100] and the 95% limits of agreement (±2 standard deviations). At least 67% of the samples had to be within ±20% limits according to cross-validation guidelines.[31,32]

Results and discussion

Method development

Assessment of adherence to antiretroviral therapy is essential to assure sufficient viral suppression and improve survival and quality of life in HIV-infected patients. While adherence in industrialized nations where TDM belongs to the standard of care generally is high, less is known about adherence to antiretroviral treatment in resource-limited countries. In such settings, the advantages of DBS samples could facilitate monitoring of antiretroviral therapy. Importantly, it has been demonstrated that antiretroviral drugs can be analysed in DBS samples, and moreover, a good correlation between plasma and DBS concentrations has been found for efavirenz and nevirapine.[20–23,33,34] However, compared with conventional plasma or serum samples, working with DBS samples entails several method-specific drawbacks. The preparation of calibrator and QC samples, as well as the extraction procedure, is more laborious. Moreover, the small amount of blood available in the DBS sample is a challenge and requires development of particularly sensitive methods. Here, we demonstrate that these challenges can, at least, in part, be overcome for the analysis of nevirapine, efavirenz and lopinavir by using a DBS autosampler system.

In the first stage of method development, mass spectrometer voltages were adjusted to maximize the peak response of the parent mass and the product ions for each compound. The best results were obtained by using the transitions 267 → 226 *m/z*, 314 → 244 *m/z* and 629 → 155 *m/z* for nevirapine, efavirenz and lopinavir, respectively. Similar transitions were used for the internal standards, with the addition of the respective amount of deuterium atoms (nevirapine-d3: 270 → 229 *m/z*, efavirenz-d5: 319 → 248 *m/z*, lopinavir-d8: 637 → 163 *m/z*). The same MS/MS transitions were also used in other published LC–MS/MS methods.[20,21,23,33] Nevirapine and lopinavir were optimized in positive ionization mode, while efavirenz was optimized in negative ionization mode. Therefore, efavirenz had to be chromatographically separated from nevirapine and lopinavir, as negative and positive ionization modes cannot be run in parallel with the employed mass spectrometer. Figure 1 illustrates that baseline separation of all analytes was

Table 1. Intra-day and inter-day accuracy and precision of nevirapine, efavirenz and lopinavir (10–10 000 ng/ml)

Analyte		Intra-day						Inter-day	
		Day 1		Day 2		Day 3		Day 1–3	
	QC level [ng/ml]	Conc. found at [ng/ml]	Accuracy ± CV [%]	Conc. found at [ng/ml]	Accuracy ± CV [%]	Conc. found at [ng/ml]	Accuracy ± CV [%]	Conc. found at [ng/ml]	Accuracy ± CV [%]
Nevirapine	10	9.4	94.1 ± 7.6	10.8	108.4 ± 6.8	10.8	107.9 ± 8.6	10.3	103.4 ± 9.8
	50	44.5	88.9 ± 8.1	47.5	95 ± 13.9	47.9	95.7 ± 6.7	46.6	93.2 ± 10.2
	500	470	93.9 ± 5.2	466	93.3 ± 4.7	481	96.1 ± 6.8	472	94.4 ± 5.5
	5000	4750	95 ± 7.4	4680	93.6 ± 5.4	4923	98.5 ± 9.9	4784	95.7 ± 7.8
Efavirenz	10	9.2	92.4 ± 8.1	9.5	95.2 ± 14.5	10.6	105.7 ± 10.4	9.8	97.8 ± 12.3
	50	46.7	93.5 ± 8.6	47.1	94.2 ± 11.5	49.6	99.2 ± 9.2	47.8	95.6 ± 9.7
	500	479	95.9 ± 5.2	475	94.9 ± 4.6	489	97.7 ± 6.6	481	96.2 ± 5.4
	5000	4767	95.3 ± 8.4	4593	91.9 ± 8	4861	97.2 ± 9	4741	94.8 ± 8.4
Lopinavir	10	9.7	96.8 ± 6.2	10.0	99.7 ± 12	10.9	109.3 ± 8.5	10.2	101.9 ± 10.3
	50	46.2	92.4 ± 10.3	45.8	91.5 ± 13.3	47.0	94.1 ± 10.1	46.3	92.7 ± 10.8
	500	486	97.2 ± 6.9	453	90.6 ± 6.3	461	92.2 ± 7.2	467	93.3 ± 7.2
	5000	4757	95.1 ± 9.4	4474	89.5 ± 8.9	4583	91.7 ± 12.2	4605	92.1 ± 10.1

Table 2. Deviation between DBS samples using 15 or 30-µl blood spot volume

Qc level [ng/ml]	Nevirapine			Efavirenz			Lopinavir		
	Concentration found at [ng/ml]			Concentration found at [ng/ml]			Concentration found at [ng/ml]		
—	15 µl	30 µl	Change [%]	15 µl	30 µl	Change [%]	15 µl	30 µl	Change [%]
10	9.4	9.5	0.7	9.2	9.7	5.1	9.7	9.5	−1.7
50	44.5	44.8	0.6	46.7	45.1	−3.4	46.2	43.2	−6.5
500	470	479	2.0	479	478	−0.2	486	455	−6.4
5000	4750	4986	5.0	4767	4790	0.5	4757	4710	−1.0

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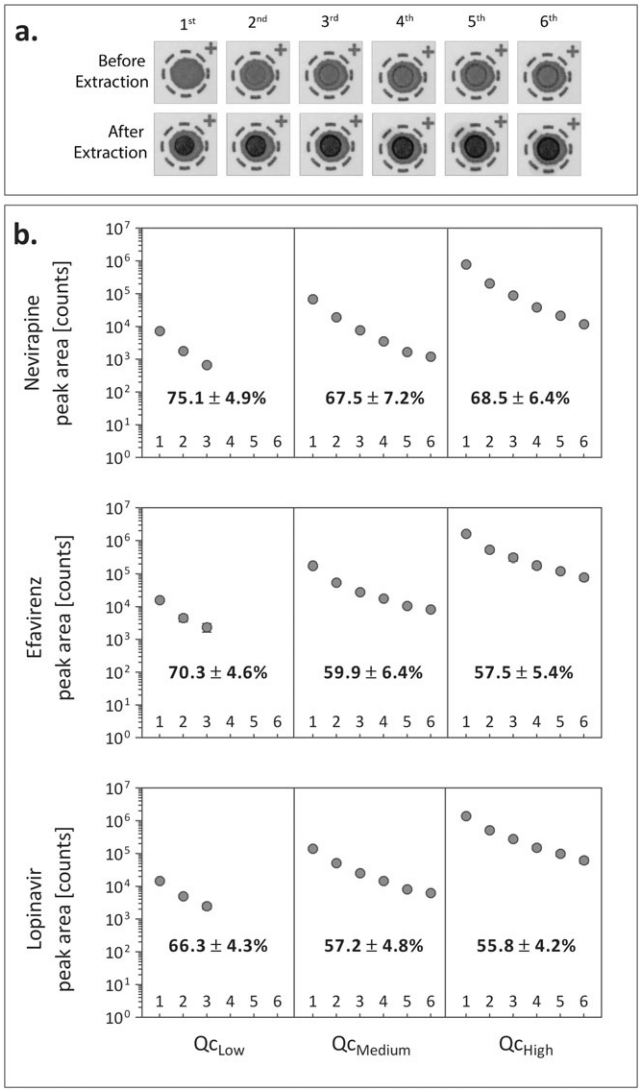

Figure 3. (a) Pictures of DBS before and after six consecutive extractions. Using the built-in camera of the autosampler, the extraction head automatically locked on the same position in the centre of the blood spot. (b) Extraction recovery of nevirapine, efavirenz and lopinavir determined at low, medium and high concentrations of four different donors. Decrease in peak area after six (medium and high QC) or three (low QC) repetitive extractions is shown. Recoveries were consistent between different subjects and over different concentrations. Bold numbers are recoveries +/− CV%.

efficiently achieved within a 3-min run time, using a core-shell pentafluorophenyl phase column. Peak symmetry was satisfactory using methanol and water supplemented with formic acid (0.1%) as mobile phase.

Further method development focused on improving the automated extraction process where the main adjustable instrument parameters are the amount of IS sprayed onto the DBS, as well as the extraction flow, extraction volume and extraction solvent composition. Parameters giving the highest signal intensities, best precisions and optimal peak shapes were selected (Fig. 2). First, different methanol : water mixtures (50 : 50, 60 : 40, 70 : 30, 80 : 20 and 90 : 10 *v/v*) were tested. A high water amount increased the risk of clogging the extraction head, as presumably more biomolecules and cellular components are removed from the DBS. Robustness of the extraction was thereby limited. Overall, a mixture of 70 : 30 methanol : water resulted in the highest signal intensities and best precision. Peak symmetry was disturbed by methanol concentrations exceeding 80% in the extraction solvent. Replacing methanol with acetonitrile did not improve extraction yield and worsened peak shapes. The extraction volume was increased stepwise from 20 to 70 μl (20, 25, 30, 40, 50, 60 and 70 μl), while the last 20 μl of each extraction were trapped in the sample loop. Signal intensities decreased with larger extraction volumes to about 80–90% of the initial value. Almost 50% is extracted within the first 10 μl of the extraction fraction. An extraction volume of 25 μl was selected because the precision was enhanced compared with 20 μl, and the signal intensity was only marginally lower. As extraction flow did not seem to have a significant impact, the standard setting of 40-μl/min flow was used to perform automated extraction. The wash cycle was optimized in order to reduce analyte carry-over. The signal in a blank sample after injection of the highest calibrator was ten times lower than the signal detected at LLOQ. Hence, carry-over of the automated extraction of the analytes is negligible.

Method validation

Selectivity and sensitivity

Selectivity of the method was tested for interfering matrix components in seven blank human DBS samples (Fig. 1). Noise level baselines of the blank samples did not show coeluting peaks at the retention time of nevirapine (1.1 min), efavirenz (1.5 min) or lopinavir (1.9 min). Moreover, internal standards that were sprayed onto blank DBS spots did not cause interfering signals. Hence, the developed method is selective for the analysis of the investigated antiretrovirals.

A sensitivity of 10 ng/ml was achieved for all analytes. Based on published data, we expect this quantification limit to be sufficient to perform therapeutic monitoring of nevirapine, efavirenz and lopinavir.[35] Figure 1 illustrates that the signal intensity at LLOQ is at least five times higher than the background noise level.

Linearity

Linearity was attained over a calibration range of 10 to 10 000 ng/ml for all analytes. Taking all validation experiments into account, the coefficient of variation (R^2) was always >0.99 for each

Table 3. Matrix effect (ME) of nevirapine, efavirenz and lopinavir in DBS samples

Concentration	Nevirapine		Efavirenz		Lopinavir	
	ME ± CV [%]	Mean ± CV [%]	ME ± CV [%]	Mean ± CV [%]	ME ± CV [%]	Mean ± CV [%]
10 ng/ml	92.2 ± 1.0	92.0 ± 0.4	52.7 ± 4.9	54.8 ± 10.4	100.6 ± 2.0	94 ± 6.2
100 ng/ml	91.5 ± 1.8	—	50.4 ± 5.4	—	91.5 ± 2.0	—
1000 ng/ml	92.1 ± 3.4	—	61.2 ± 11.0	—	89.7 ± 2.0	—

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Table 4. Stability of nevirapine, efavirenz and lopinavir evaluated under different conditions. DBS samples at a concentration of 500 ng/ml were used

Condition	Nevirapine			Efavirenz			Lopinavir		
	Concentration [ng/ml]	Accuracy ± CV [%]	Change in concentration [%]	Concentration [ng/ml]	Accuracy ± CV [%]	Change in concentration [%]	Concentration [ng/ml]	Accuracy ± CV [%]	Change in concentration [%]
RT	519.5	103.9 ± 3.6	—	481.6	96.3 ± 6.1	—	532.7	106.5 ± 3.3	—
4 weeks, 4 °C	532.4	106.5 ± 7.3	2.5	511.1	102.2 ± 8.8	6.1	537.2	107.4 ± 8.2	0.8
4 weeks, −20 °C	517.4	103.5 ± 7.2	−0.4	477.7	95.5 ± 8.2	−0.8	559.2	111.8 ± 6.7	5.0

analyte. The upper limit of quantification also encompasses high therapeutic through concentrations.[11,12,36,37] This is especially important for the automated extraction of DBS, as dilution of the DBS samples cannot be easily performed. Thus, clinically occurring concentrations can be quantified by linear regression within the chosen calibration range.

Accuracy and precision

Intra-day and inter-day accuracy and precision data are summarized in Table 1. QC samples were derived from seven donors. Intra-day precisions of less than 13.9%, 14.5% and 13.3% were found for nevirapine, efavirenz and lopinavir respectively. Mean intra-day accuracies were between 88.9 and 108.4% for nevirapine, 91.9 and 105.7% for efavirenz and 90.6 and 109.3% for lopinavir. Inter-day precisions were ≤12.3% for all three analytes. On all occasions, not more than two out of the seven QC samples per concentration were outside the 85–115% (LLOQ: 80–120%) limits.

In addition, concentrations of QC samples differed by less than 6.5% when 30 μl instead of 15 μl of blood was spotted onto cards (Table 2). Hence, the applied blood volume produced reproducible, volume-dependent spot areas and therefore did not affect accuracy and precision of the analysis. Overall, the results were consistent with conditions specified by regulatory guidelines.[29]

Recovery and matrix effect

The relative recovery and matrix effect was determined in four different donors at low, medium and high QC levels (Fig. 3). In addition, the recovery of the analytes was assessed by using aqueous analyte solutions spotted onto the filter paper ('dried water spots'). The highest mean recovery was achieved for nevirapine with 70%, followed by efavirenz (63%) and lopinavir (60%). A bias in recovery of less than 7.2% was determined between subjects, indicating high consistency in the sample extraction process. Recovery at low concentration was overestimated by approximately 10% compared with medium and high concentration samples, as the limit of quantification was already reached after three instead of six consecutive extractions. The recovery of dried water spots was >97%, indicating that blood acts as an extraction barrier.

The signal intensity of nevirapine and lopinavir was only suppressed by the DBS matrix by about 8% and 6% respectively (Table 3). However, a significant suppressive matrix effect was observed for efavirenz, where the signal was reduced to approximately 50% due to the biological matrix. Importantly, the matrix effect was independent of concentration, as its deviation was ≤10.4% over the tested concentration range and it did not vary between subjects (CV ≤ 11.0%). Hence, the matrix of different donors did not alter the reliability of the method. Online solid phase purification of DBS extracts, via column switching or by more thorough separation of efavirenz and blood ingredients, may eliminate suppressive matrix effects.[38,39] In summary, the automated extraction was reproducible and delivered consistent matrix effects.

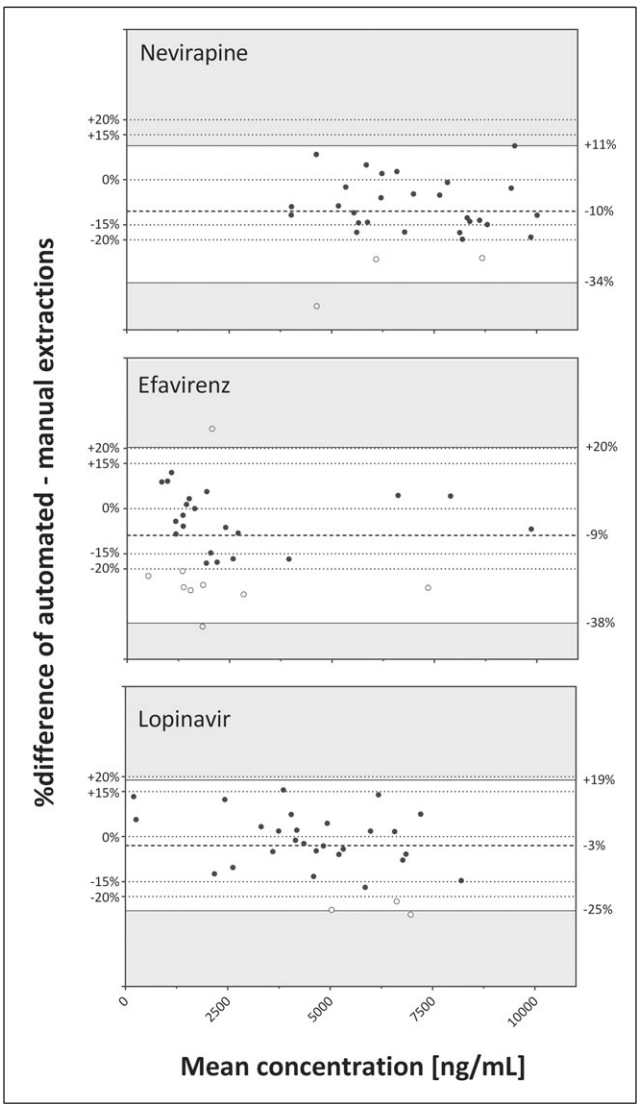

Figure 4. Bland–Altman plots of nevirapine, efavirenz and lopinavir were generated for automated and manual DBS extractions. Values were coloured grey if the %difference of the two extraction methods was less than ±20%; otherwise, values were coloured white. The white plot area illustrates the 95% limits of agreement. The black dashed line describes the mean percentage difference between automated and manual extraction.

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Stability

Stability data are summarized in Table 4. The data implied that analytes were stable in DBS for 4 weeks at −20 °C or 4 °C, as the change in concentration compared with a freshly prepared DBS sample was less than 6.1%. These results are in line with stability data published in previous studies.[23,33,40,41] Therefore, DBS samples containing nevirapine, efavirenz and lopinavir are not likely to be degraded by storage in the fridge for 1 month.

Method application

Ninety DBS samples (30 DBS per analyte) collected in the framework of a drug adherence study in Ifakara (Tanzania) were analysed after manual extraction as well as after automated extraction using the DBS-MS 500 autosampler. Nevirapine concentrations in DBS were between 3360 and 10 800 ng/ml, efavirenz concentrations were between 262 and 10 200 ng/ml, and lopinavir concentrations were between 41.8 and 8 800 ng/ml; thus, the selected calibration range was suitable for the quantification of the collected TDM samples. Sample dilution was therefore not necessary, which is relevant because it cannot be easily performed through automated extraction.

Overall, the results obtained after automated and manual extractions were in good agreement (Fig. 4). The mean bias of automated to manual extractions was −10.5% for nevirapine, −8.9% for efavirenz and −3.0% for lopinavir. Ninety-five per cent limits of agreement were narrow with a range of about ±25% (nevirapine: −32.3 to +11.3%; efavirenz: −38.1% to +20.4%; lopinavir: −24.7% to +18.8%). Consequently, when comparing automated and manually extracted samples, 70% or more of the respective samples showed a deviation of less than 20%, according to cross-validation guidelines.[31,32] No concentration-dependent trend was observed between the ratios of the two extraction methods across the entire concentration range. Overall, automated extraction was five times more sensitive than manual extraction, which is mainly due to DBS being extracted with less solvent through the automated sample workup (25 *vs* 150 μl) resulting in more concentrated samples. Both extraction procedures achieved similar results, and the data obtained were in agreement with afore mentioned criteria.[31] A thorough clinical validation including the comparison of plasma and DBS samples will be published elsewhere.

Conclusions

We developed and validated an innovative LC–MS/MS method by using on-line DBS extraction for the quantification of three frequently used antiretroviral drugs. The short time of approximately 3 min to extract and analyse one DBS sample makes the method suitable for handling larger sample loads. Automated DBS extraction entails specific challenges regarding method validation and requires novel approaches to assess, e.g. extraction recovery and matrix effects. The calculated interindividual recoveries and matrix effects were very consistent, and validation parameters were in accordance with regulatory guidelines. The method was successfully applied to analyse TDM samples from a field study. The results obtained by automated and manual extraction were comparable; however, sample processing time and method sensitivity were improved by the automated extraction procedure. Nevertheless, a comprehensive clinical validation comparing DBS with plasma samples is required before the developed method can be implemented for TDM in daily practice.

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Conflict of interest

Stefan Gaugler is an employee of CAMAG (Muttenz, Switzerland). None of the other authors reports any conflict of interest regarding this study.

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