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Development and validation of a fully automated online human dried blood spot analysis of bosentan and its metabolites using the Sample Card And Prep DBS System

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ABSTRACT

This paper describes the development and validation of a liquid chromatography (LC)–electrospray ionization tandem mass spectrometry assay for the fully automated simultaneous determination of bosentan, a dual endothelin receptor antagonist used in the treatment of pulmonary arterial hypertension, and its three primary metabolites hydroxy bosentan (Ro 48-5033), desmethyl bosentan (Ro 47-8634), and hydroxy desmethyl bosentan (Ro 64-1056) in human dried blood spots (DBS) by use of the Sample Card And Prep (SCAP) DBS System. The system enabled the online extraction of compounds from filter paper cards without the need for punching and sample pretreatment. This was realized by automatic introduction of DBS sample cards into the LC flow via a pneumatically controlled clamp module. Using a three-column setup comprised of two pre columns for successive online DBS sample cleanup and a Synergi™ POLAR-RP C_{18} analytical column for chromatographic separation under gradient conditions with a mobile phase A consisting of 1% acetic acid and a mobile phase B consisting of 1% acetic acid in methanol/2-propanol (80/20, v/v). MS/MS detection was performed in the positive multiple reaction monitoring mode using a Sciex API 4000 triple quadrupole LC–MS/MS system equipped with a TurboIonSpray™ source. The total run time was 9.0 min. The individual phases of online human DBS analysis were synchronized by automated valve switching. The analytical method was shown to be sensitive and selective with inter-day accuracy and precision of 91.6–108.0% and 3.4–14.6%, respectively, and it exhibited good linearity ($r^2 \geq 0.9951$ for all analytes) over the concentration range of 2 ng/mL (5 ng/mL for Ro 47-8634)–1500 ng/mL. The analytes were stable in human DBS over 3.5 months at ambient temperature and accurate and precise results were obtained when using a blood spot volume between 20 and 30 μL. Furthermore, no apparent (−8.9 to 12.6%) impact of hematocrit values ranging from 0.35 to 0.65 was observed on the quantification of the analytes. The system allowed very good recoveries of all analytes, between 83.0% and 92.3% for bosentan, between 94.4% and 100% for Ro 48-5033, between 98.0% and 100% for Ro 47-8634, and between 94.3% and 100% for Ro 64-1056. The validation demonstrated that the SCAP DBS System provides a robust automated platform for DBS analysis.

Abbreviations: cps, counts per second; CV, coefficient of variation; DBS, dried blood spot; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FDA, Food and Drug Administration; ISTD, internal standard; LC, liquid chromatography; LLOQ, lower limit of quantification; MF, matrix factor; MP, mobile phase; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; QC, quality control; PAH, pulmonary arterial hypertension; r^2, correlation coefficient; RP, reversed-phase; SCAP, Sample Card And Prep; ULOQ, upper limit of quantification.

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1. Introduction

Dried blood spot (DBS) analysis has been known for more than four decades. The technology has especially been used for therapeutic drug monitoring and as a screening method for metabolic disorders [1–3]. Recently DBS has experienced growing popularity as an alternative and very promising sampling procedure in bioanalytical, clinical, and pharmaceutical applications. The applicability of DBS in modern drug development was initially demonstrated by Beaudette and Bateman [4]. To date, DBS-LC–MS/MS has emerged as an important technology for quantitative analysis of samples from both preclinical and clinical studies [5–7]. This development has also been enabled by the recent technological advancements in MS in terms of sensitivity.

DBS has several advantages over conventional plasma, serum, and whole blood analysis [8–10]. The low amount of test material needed, typically 10–30 μL, makes DBS analysis ideally suited for neonatal and infant screening. It allows a less invasive sample collection procedure, e.g., by finger or heel prick as compared to conventional venipuncture. With regard to preclinical investigations the low blood volume needed affords a reduced number of rodents required per study. This simplifies the logistics for DBS sample processing. In contrast to conventional liquid sampling methods, refrigeration and freezing of samples is not necessarily needed for DBS sample handling, storage, and transport. This provides a high flexibility with regard to study sample management, particularly in tropical and subtropical regions, e.g., in the conduct of field studies. Furthermore the risk of infection by pathogens such as HIV or hepatitis is significantly reduced [3,11].

Conventional DBS analysis also exhibits two drawbacks, namely the requirement for (manual) punching of blood spots and offline extraction. These restrictions make DBS analysis less attractive and, at the end, not appropriate for high throughput applications. DBS punching instruments, which are able to automatically punch DBS samples into microtitration plates, only enable the semi-automated analysis of DBS specimens. In order to avoid offline extraction of DBS samples several new concepts for the direct analysis of DBS have been developed [12–18]. Abu-Rabie and Spooner initially reported a thin-layer chromatography mass spectrometer interface having the potential to be an effective tool for the direct analysis of drugs from DBS samples [19]. In 2009 Déglon et al. demonstrated the feasibility and applicability of an online DBS procedure, called online desorption of DBS, which allowed the direct analysis of a DBS sample. The DBS was set into a homemade desorption cell that was integrated into a conventional liquid chromatography mass spectrometry (LC–MS) system [20]. The authors then expanded the strategy by coupling the process with an automated system of valves to set five filter papers online [21]. Very recently, the authors reported a prototype for the online DBS analysis, which allows the successive extraction of 30 DBS samples with good results in terms of precision, trueness, and linearity [22]. However, prior to analysis each DBS needs to be manually punched out and internal standard (ISTD) has to be added using a volumetric pipette. Although this prototype seems to be a very promising development it on the other hand clearly demonstrates the need for full automation of the DBS workflow in order to meet demands for the analysis of large sample sets in clinical pharmacology.

There are several options of applying ISTD into the automated extraction process for DBS analysis. An ISDT is a close structural analogue of the analyte being quantified, preferably a stable isotope labeled analogue. The ISTD is added in equal concentration to all calibrations standards, quality control samples, and study samples to compensate for fluctuations in the analyte response during sample preparation and analysis. Since co-eluting compounds from the matrix may interfere with the quantification of the analytes the addition of a stable isotope labeled ISTD is desirable. This is because of the similarities in chemical properties and elution times of the stable isotope labeled ISTD relative to the analytes. With regard to the techniques incorporating the ISTD into the elution solvent, it is argued that the ISTD does not compensate for any variability during the extraction process [23]. This is because the ISTD is not added prior to the extraction process and, therefore, is not entirely incorporated into the matrix components and the sample paper. Nevertheless, the incorporation of ISTD into the extraction solvent can easily be accomplished and provides reproducible results. In a previous study the newly developed Sample Card And Prep (SCAP) DBS System was used for the determination of the antiviral drug oseltamivir (Tamiflu®) and its active metabolite oseltamivir carboxylate in DBS and shown to allow the online extraction and quantification of these pharmaceutical compounds in a fully automated fashion that is competitive with conventional liquid analysis [24]. The authors demonstrated that sensitivity, precision, and accuracy in DBS were comparable to standard plasma assays. Furthermore, automation of the analytical process ensured workload reduction (e.g., no tedious punching and offline extraction necessary) and thus a sample throughput comparable with that of conventional liquid sample analysis, i.e., parallel processing in the 96-well format.

Besides incorporating the ISTD into the elution solvent, several other options of ISTD application into the automated extraction process for DBS analysis are currently being discussed. The addition of ISTD to the liquid blood sample prior to application on the filter paper would ensure that it is fully incorporated into the matrix along with the analyte. However, as pre-treating blank paper substrate with ISTD, this approach is logistically not practicable in multicenter studies and would negate the straightforwardness of the sampling process.

Another option is the ISTD application to the DBS prior to extraction. This procedure would potentially ensure full interaction of ISTD with the matrix and the filter paper substrate. However, several aspects need to be addressed to ensure reproducibility of this technique. This is the homogeneous application of the ISTD solution across the DBS sample. Furthermore, it has to be demonstrated that the application of the ISTD does not adversely affect the distribution of the analyte. Finally, the risk of ISTD contamination to adjacent blood spots has to be evaluated. Abu-Rabie et al. [23] have initially investigated the use of a piezo electric spray technology as a method of adding ISTD solution to DBS samples for use in quantitative bioanalysis. The CAMAG DBS-MS 500 extraction system for a fully automated DBS direct analysis was recently introduced [25]. It utilizes an integrated spraying module for the application of the ISTD prior to online extraction of the DBS card [26]. As the SCAP DBS System described here, the CAMAG DBS-500 system has an integrated camera for optical DBS card and spot recognition and enables the automated robotic handling of up to 500 cards. The extraction process of the CAMAG DBS-MS 500 system either involves a sample loop or solvent can be flushed through a trapping column before MS detection.

Here, the SCAP DBS System was successfully evaluated by validation of an analytical method for the determination of bosentan and its primary metabolites (Fig. 1) in human DBS. As an endothelin receptor antagonist bosentan has emerged as an important drug in the therapy of pulmonary arterial hypertension (PAH), an incurable disease defined by an increase in pulmonary vascular resistance, which ultimately can lead to death [27,28]. In human blood bosentan is >98% bound to plasma proteins, mainly albumin, and does not penetrate into erythrocytes. It is metabolized to three metabolites. The major metabolite in plasma, Ro 48-5033, is pharmacologically active. It is generated by hydroxylation at the *tert*-butyl group of bosentan. The two other metabolites are Ro 47-8634, which is formed by *O*-demethylation of the phenolic methyl ether, and Ro 64-1056, the product of both demethylation and hydroxylation of

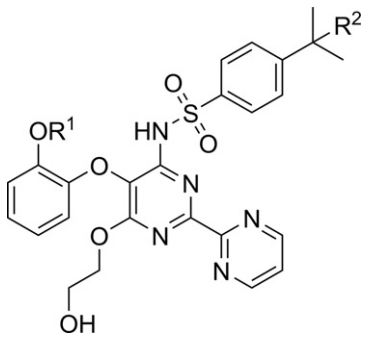

Fig. 1. Chemical structures of the analytes used to generate the reported results. Bosentan: $R^1 = CH_3$ and $R^2 = CH_3$; Ro 48-5033: $R^1 = CH_3$ and $R^2 = CH_2OH$; Ro 47-8634: $R^1 = H$ and $R^2 = CH_3$; Ro 64-1056, $R^1 = H$ and $R^2 = CH_2OH$.

bosentan [29]. To date bosentan and metabolite levels have been determined by plasma analysis [30]. However, for the determination of bosentan and metabolite concentrations in newborns during PAH treatment this may be challenging because of the relatively high volume of blood needed per time point (ca. 500 μL of blood). The current work describes a novel DBS-LC–MS/MS strategy that allows to simultaneously monitor bosentan and metabolite concentrations in human blood in a fully automated way. The assay was validated following the Food and Drug Administration (FDA) guidance for bioanalytical method validation [31].

2. Materials and methods

2.1. Chemicals and materials

HPLC grade acetonitrile and methanol were purchased from Acros (Geel, Belgium). HPLC grade water and 2-propanol were from Fisher Scientific (Leicestershire, UK). Dimethylsulfoxide (purissimum) (DMSO) and acetic acid (96%) were from Merck (Darmstadt, Germany).

Bosentan, Ro 48-5033, Ro 47-8634, and Ro 64-1056 as well as the corresponding d4-deuterated ISTDs were supplied by Actelion Pharmaceuticals Ltd (Allschwil, Switzerland).

FTA™ DMPK-A cards were purchased from GE Healthcare (Dassel, Germany).

Blank human blood containing K3-EDTA as anticoagulant was obtained from the Blutspendezentrum SRK, Kantonsspital Aarau, Switzerland.

All other reagents were of analytical grade.

2.2. Preparation of stock solutions, calibration standards, and quality control samples

The stock solutions of bosentan, Ro 48-5033, Ro 47-8634, and Ro 64-1056 with concentrations of 1 mg/mL were prepared by dissolving at least 5 mg of each substance in DMSO. These stock solutions were used to prepare working standard solutions with concentrations ranging from 75.0 to 0.10 μg/mL by appropriate dilutions with DMSO. The working standards were used for the preparation of calibration standards by spiking whole blood to obtain final concentrations of 2 ng/mL (Ro 47-8634: 5 ng/mL), 5 (10), 10 (20), 24 (48), 96, 240, 600, and 1500 ng/mL.

The corresponding ISTD stock solutions (each 1 mg/mL) were prepared by dissolving at least 1 mg of each substance in DMSO. A combined ISTD working solution was prepared by diluting aliquots of the ISTD stock solutions with H_2O to obtain final concentrations of 25 ng/mL for d4-bosentan and 12.5 ng/mL for d4-Ro 48-5033, d4-Ro 47-8634, and d4-Ro 64-1056.

The stock solutions and working solutions were stored in the dark at 4 °C.

Quality control (QC) samples at concentrations of the lower limit of quantification (LLOQ) (2 ng/mL for bosentan, Ro 48-5033, and Ro 64-1056; 5 ng/mL for Ro 47-8634), QC_{low} (6 ng/mL and 15 ng/mL, respectively), QC_{mid} (120 ng/mL) and QC_{high} (1250 ng/mL) were prepared analogously to the calibration standards.

2.3. Blood spotting

For the preparation of human EDTA blood spots, 25 μL of blank or spiked blood were spotted onto DMPK-A cards by use of a calibrated pipette (Gilson, Mettmenstetten, Switzerland) and left to dry for at least 2 h at ambient temperature. After drying, cards were stored in the dark at ambient temperature in plastic zip-closure bags containing desiccant packs (1 card and desiccant pack per ziploc bag) until analysis. The integral transparent strip of each desiccant packet permitted observation of the contained adsorbent and allowed desiccants effectively showing when moisture had been adsorbed and desiccant had to be replaced (which was not necessary throughout the validation). The storage temperature, monitored by a data logger, was 23 ± 3 °C. The relative humidity was monitored by a hygrometer and was 45 ± 15%. The diameter of a 25 μL DBS was approximately 9 mm.

2.4. Instrumentation

The validation was conducted with a SCAP DBS System (Prolab Instruments GmbH, Reinach, Switzerland) (Fig. 1, supplementary data) coupled to an LC–MS/MS system, which was comprised of the following components: an API 4000 triple quadrupole instrument with a TurboIon™ Spray interface (MDS SCIEX, Rotkreuz, Switzerland), two extraction pumps (L-6200A and L-6200, Merck Hitachi, Dietikon, Switzerland), two dilution pumps (L-6200A, Merck Hitachi), and two analytical gradient pumps (LC-10AD$_{VP}$, Shimadzu, Reinach, Switzerland).

The SCAP DBS System was controlled with the SCAP DBS software (Prolab Instruments GmbH). Data acquisition, processing, and calculation were performed with Analyst software (version 1.4.1; MDS SCIEX). The synchronization of the SCAP DBS System with the LC–MS/MS system was done via an out expansion box (CTC Analytics AG, Zwingen, Switzerland).

2.5. The SCAP DBS System

The SCAP DBS System enables a variable, permanent flow of LC solvents through the filter paper of the DMPK-A card during the analysis. The system is based on the robotics of a PAL autosampler (CTC Analytics AG). For the realization of the SCAP DBS System the PAL autosampler sample trays were replaced by custom-made DBS card racks with a total capacity for 160 DBS cards. Furthermore, the syringe adapter and plunger holder were replaced by a robotic gripper, which executes the transfer of DBS cards from the sample rack to the pneumatically controlled clamp module according to the sample queue. Via the clamp module DBS cards were integrated into the LC flow path for online extraction. The clamp module contained a piston that was activated by pressurized air (0.4 MPa). Locking of the DBS cards during analysis was achieved by two interchangeable clamp adapters. The dilutor module of the SCAP DBS System enabled the online addition of ISTDs. Three integrated switching valves, one ten-port valve and two 6-port valves, controlled the LC flows and synchronized the addition of ISTDs.

The SCAP DBS System utilizes the integrated camera for visual DBS card and spot recognition. Before locking a DBS card in the clamp module a picture of the gripped DBS card is taken and electronically saved. This enables the online DBS card identification

Table 1
MS parameters for bosentan, Ro 47-8634, Ro 48-5033, Ro 64-1056 and corresponding internal standards.

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Collision energy (V)	Dwell time (ms)
Bosentan	552.2	202.1	49	50
Ro 47-8634	538.2	188.8	44	50
Ro 48-5033	568.2	202.1	47	50
Ro 64-1056	554.3	188.8	51	50
d4-Bosentan	556.2	202.1	49	25
d4-Ro 47-8634	542.2	188.8	44	25
d4-Ro 48-5033	572.2	202.1	47	25
d4-Ro 64-1056	558.3	188.8	51	25

(e.g., via a bar code) and the accurate location of each spot position by the SCAP DBS software. Imprecisely spotted DBS samples are identified and an automatic *x*, *y* offset to the robotic arm is performed before DBS card clamping and extraction. This ensures DBS sample extraction exactly from the center of each spot. In addition, via a user pre-defined offset, multiple extractions from the same spot at different spot positions can be carried out enabling the reanalysis of DBS samples. After the online extraction of a spot (position) a second picture of the DBS card is automatically taken and electronically saved allowing the user to also manually inspect each extraction.

2.6. Fully automated analysis of dried blood spots

The process of the fully automated LC–MS/MS analysis of DBS samples is divided into four phases (Fig. 2, supplementary data). A three-column setup consisting of two pre columns and an analytical reversed-phase (RP) column enabled the online analyte extraction, sample cleanup, and chromatographic separation. Final quantitative determination of compound levels was achieved by online ESI-MS/MS.

At the start of each DBS analysis the 10-port valve and the two 6-port valves were automatically set into 'stanfigd by' mode (connected positions of 10-port valve: 2-3, 4-5, 6-7, 8-9, 1-10; connected positions of 6-port valves: 2-3, 4-5, 1-6). The DBS card was transferred to the clamp module and the loop filled by the dilutor with 20 μL of ISTD solution (full-loop injection) (Fig. 2A, supplementary data). Following automatic switching of the 10-port valve into 'active' state (connected positions: 1-2, 3-4, 5-6, 7-8, 9-10), the extraction of the DBS and subsequent loading of analytes and ISTDs onto the first pre column (LiChrospher 100 RP18 ADS, 25 mm × 2 mm, 25 μm, operated at room temperature; Merck, Darmstadt, Germany) was carried out by flushing extraction solution, methanol–water (50:50, v/v), through the loop and the DBS at a flow rate of 250 μL/min (extraction pump 1) for 2 min (Fig. 2B, supplementary data). For quantitative binding of analytes onto the first pre column the flow was diluted with mobile phase (MP) A, consisting of water–acetic acid (100:1, v/v), at a flow rate of 2000 μL/min (dilution pump 1). The addition of MP A reduced the organic content of the LC flow from 50% to 5.5%. At $t = 2$ min the 10-port valve switched back to 'stand by' mode thus enabling the washing of the tubing via the first pre column into waste 1 for 1 min. Between $t = 3$ min and $t = 4.5$ min the transfer of analytes from pre column 1 to pre column 2 (CC 8/4 Nucleosil 100-5 C18 HD, 5 μm, kept at 50 °C; Macherey-Nagel, Oensingen, Switzerland) in forward flush mode was carried out (Fig. 2C, supplementary data). For this step the first 6-port valve switched to 'active' state (connected positions: 1-2, 3-4, 5-6) and extraction pump 2 provided MP B, consisting of methanol–2-propanol–acetic acid (80:20:1, v/v/v), at a flow rate of 250 μL/min. The addition of MP A to the LC flow at 3000 μL/min (dilution pump 2) enabled the binding of analytes on pre column 2. Simultaneously, pre column 1 was flushed with organic washing solution, 2-propanol, directly into waste 1 using extraction pump 1 at a flow rate of 3000 μL/min. For their chromatographic separation and online MS/MS detection, analytes were eluted in back flush mode between 4.5 and 6.0 min from pre column 2 to the analytical column (Synergi POLAR-RP C18, 50 mm × 2 mm, 4 μm, kept at 30 °C; Phenomenex, Brechbühler, Switzerland), which was directly interfaced to the ESI source of the mass spectrometer (Fig. 2D, supplementary data). The 10-port valve and the first 6-port valve switched back to 'stand by' state, while the second 6-port valve switched to 'active' state.

Elution from pre column 2 onto the analytical column and chromatographic separation were performed using the analytical gradient pump system. During elution from pre column 2 onto the analytical column the gradient transitioned from 10 to 35% MP B at a flow rate of 300 μL/min during the first 0.4 min at which it was held for 1.1 min. For subsequent chromatographic separation the gradient transitioned from 35 to 45% MP B during 0.2 min and from 45 to 85% over 0.4 min at a flow rate of 300 μL/min. The gradient was held for 0.5 min at 85% MP B and increased to 98% in 0.1 min where it was held for 0.9 min at the flow rate of 300 μL/min. While keeping the mobile phase composition constant, the flow rate was increased to 750 μL/min for additional 2.6 min. Then the gradient transitioned back to 10% MP B in 0.1 min. Re-equilibration was performed at 10% MP B for 2.2 min during which the flow rate was kept at 750 μL/min for the first 1.7 min and then decreased to 300 μL/min for the remaining 0.5 min. In addition, between 5.1 and 6.0 min pre column 1 was flushed with aqueous washing solution, water–acetic acid (100:1, v/v), directly into waste 1 using extraction pump 1 at a flow rate of 3000 μL/min. Simultaneously to the analytical separation re-conditioning of the front part of the LC system was enabled by synchronized switching of all valves to 'stand by' state. This facilitated washing of the tubing and pre column 1 with methanol–water (50:50, v/v) and flushing of pre column 2 with MP B directly into waste 2. At the end of the analysis the clamped DBS card was released and transferred back to the sample rack. The total cycle time for one analytical run was 9.0 min.

Mass spectrometric detection was synchronized with the start of the elution of analytes from pre column 2 towards the analytical column at $t = 4.5$ min and performed with positive multiple reaction monitoring (MRM) for which the following parameters were set for all compounds: curtain gas (nitrogen): 30 (arbitrary units); ion source gas 1 and 2 (nitrogen): 50; collision gas (nitrogen): 11; ionspray voltage: 5000 V; temperature: 650 °C; declustering potential: 90 V; entrance potential: 11 V; collision cell exit potential: 12 V; resolution Q1 and Q3: high. For each compound, one MRM transition was recorded for quantification, selected on the criteria of intensity and selectivity obtained for the respective fragment ion. The individual settings are listed in Table 1.

2.7. Validation process

The validation was conducted according to the FDA guidance for bioanalytical method validation [31]. During the validation process the following parameters were evaluated.

2.7.1. Whole blood stability

To evaluate whether the analytes are prone to enzymatic degradation that could take place during the collection and handling of blood, human liquid EDTA blood was spiked with the analytes at the QC_{low} and QC_{high} level and kept for 6 h at ambient temperature before being stored at $6 \pm 4\,^{\circ}C$ for two days. Whole blood stability was determined by spotting QC_{low} and QC_{high} samples onto DMPK-A cards after storage for 6 h at ambient temperature and after two-day storage at $6 \pm 4\,^{\circ}C$. Six QC_{low} and six QC_{high} samples were analyzed against freshly prepared calibration standards.

2.7.2. Linearity/sensitivity

The analysis of calibration samples was performed by internal standardization using the deuterated analogs of the analytes. Calibration curves were constructed from the results of eight concentration levels over the specified range. Calibration curves were fitted by least-squares linear regression using 1/concentration^2 ($1/x^2$) as the weighting factor.

The lower limit of quantification (LLOQ) was the lowest concentration of the calibration curve that could be measured with acceptable accuracy (≤20%) and precision (coefficient of variation (CV) ≤20%).

2.7.3. Selectivity

Selectivity of the method was determined by analyzing six different batches of blank human DBS samples without adding ISTDs. The mean interference had to be below 1/5 of each of the analyte mean responses at the respective QC_{LLOQ} level (2 ng/mL; 5 ng/mL for Ro 47-8634).

In detail, 12 blank DBS samples of one matrix source were analyzed, six at the QC_{low} and QC_{high} level. The concentrations of the ISTDs were equal to those used in regular DBS analysis (QC_{reg}). In the comparison experiment blank DMPK-A cards onto which no blood had been spotted were analyzed accordingly.

For the quantitative measurement of the matrix effect the matrix factor (MF) was determined [32]. The MF was defined as the ratio of the analyte or ISTD peak response in the presence of blood matrix ions (DBS blank sample) to the analyte or ISTD peak response in the absence of blood matrix ions (blank DMPK-A card):

$$\mathrm{MF} = \frac{\text{Peak response}_{\text{DBS blank sample}}}{\text{Peak response}_{\text{Blank DMPK-A card}}}$$

The CV% from replicate measurements had to be ≤15%.

2.7.7. Recovery

The efficiency of the extraction procedure was determined by the analysis of six replicates of human DBS spiked with the analytes at the QC_{low}, QC_{mid}, and QC_{high} level. During recovery testing a DBS sample was consecutively extracted and analyzed three times at the same spot position. For this purpose a regular extraction and subsequent LC–MS/MS analysis (entire run cycle) of a DBS sample was performed. Without removing the DBS card from the clamp module, the same blood spot was re-extracted and analyzed twice at the identical position during two further entire run cycles. The ratio of the analyte peak response of the first extraction and the sum of analyte peak responses detected during the first, second, and third extraction defined the individual recoveries. The CV% from replicate measurements had to be ≤15%:

$$\text{Recovery}\,(\%) = \frac{\text{Peak response}_{\text{First extraction}}}{\text{Peak response}_{\text{First extraction}} + \text{Peak response}_{\text{Second extraction}} + \text{Peak response}_{\text{Third extraction}}} \times 100$$

2.7.4. Specificity

To demonstrate the specificity of the method, six lots of blank human blood were spiked with bosentan and its metabolites at the level of the LLOQ, subsequently spotted onto DMPK-A cards and analyzed as QC_{LLOQ} samples.

The accuracy of the analysis of the six lots had to be within ±20% of the nominal concentration. The precision, expressed as CV%, had to be within 20%.

2.7.5. Accuracy and precision

Accuracy and precision were evaluated on three separate days by replicate analysis of six QC samples at each of the concentration levels QC_{LLQC}, QC_{low} (6 ng/mL; 15 ng/mL for Ro 47-8634), QC_{mid} (120 ng/mL), and QC_{high} (1250 ng/mL).

Intra-day accuracy and precision were determined for each day (number of samples per level: $n = 6$); inter-day accuracy and precision were calculated using all samples at each concentration level analyzed over three days ($n = 18$).

For the accuracy to be considered acceptable, the mean value had to be within 15% of the actual value except at LLOQ, at which it should not deviate by more than 20%. The precision determined at each concentration level had to be within 15% of the CV except for the LLOQ, at which it should not exceed 20% of the CV.

2.7.6. Matrix effect

Due to the automated online extraction of the analytes from the clamped DBS card a conventional post-fortification experiment of blank human DBS samples after extraction could not be conducted for the evaluation of the matrix effect in MS/MS detection. In an alternative approach, blank DBS samples were “extracted” with an aqueous neat solution containing all analytes and ISTDs.

2.7.8. Effect of hematocrit

Human EDTA blood (hematocrit level of 0.47) was centrifuged to produce fractions containing blood cells and plasma. Blood samples with hematocrit values of 0.37 and 0.67 were prepared by adding the appropriate volumes of plasma to the packed blood cells, followed by gentle mixing. Blood samples containing the analytes at the low and high QC level were prepared on the day of analysis at each hematocrit level. The liquid added during spiking of analytes into the blood portions reduced the hematocrits to 0.35, 0.45, and 0.65, respectively. After spotting onto DMPK-A cards, the samples were analyzed along with a set of calibration standards prepared at hematocrit of 0.45. The average analyte concentrations for replicates of six at hematocrit 0.35 and 0.65 were calculated and the difference (expressed as a percentage) calculated from values at hematocrit of 0.45 at the QC_{high} and QC_{low} level. The CV% from replicate measurements had to be ≤15%.

2.7.9. Carryover

Carryover of bosentan and metabolites was assessed by injecting a test sequence consisting of one blank DBS sample followed by one upper limit of quantification (ULOQ) sample, and a DBS blank sample. The ULOQ sample represented the highest concentration of the calibration curve (1500 ng/mL). The test sequence was repeated three times. The carryover was determined by comparing the analyte peak responses in the DBS blank sample with those in the ULOQ sample at the retention times of the analytes.

2.7.10. Robustness/influence of blood spot volume

Since different blood spot volumes may result in different measured analyte concentrations [33,34], the impact of the blood

spotting volume on the DBS analysis was investigated. For this purpose six QC samples at the low and high level were spotted onto DMPK-A cards using increasing volumes (20 μL, 25 μL, and 30 μL). After drying, the samples were measured along with a set of calibration standards, for which the spotted blood volume was 25 μL. The nominal concentrations of the QC_{low} and QC_{high} samples were used as reference.

2.7.11. Long-term stability of DBS

For the evaluation of the long-term stability, 3 sets of QC samples consisting of six replicates at the QC_{low} and QC_{high} level were prepared. After drying of the spots for at least 2 h at ambient temperature one set was analyzed on the day of preparation. The means of the measured concentrations at each level were used as references for the stored QC samples. The second and third QC sample set were stored light protected at ambient temperature for 5 weeks and 3.5 months, respectively, before being analyzed against freshly prepared calibration standards.

2.7.12. Stock solution stability

Stock solutions of bosentan and the metabolites were kept for 6 h at ambient temperature before being stored at 6 ± 4 °C for 5 months. After completion of the storage period, the peak areas of samples prepared using stored stock solutions were compared with the peak areas of samples prepared from freshly prepared stock solutions. The samples were prepared by diluting the individual stock solutions with DMSO followed by spiking blood in six replicates to obtain analyte concentrations in the upper range of the calibration curve.

The deviation between the mean areas of samples made from stored and freshly prepared stock solutions had to be within ±5% for each analyte.

3. Results and discussion

3.1. Method development

During the development of the assay a significant sensitivity loss and gradually deteriorating chromatography with regard to peak shapes and widths were observed in the course of approximately 40 successive injections. In addition, irreversible clogging of columns and the PEEK capillary between the clamp adapter and the 10-port valve occurred which resulted in a backpressure value exceeding the pump limits. The accumulation of hydrophobic matrix components such as proteins and phospholipids was most likely the cause for the continuous decrease of analytical performance and clogging during repeated injections. Therefore, extensive organic and aqueous washing phases were introduced into the LC method. The introduction of 2-propanol, a solvent exhibiting very strong eluting properties in RP chromatography, was found to be crucial to significantly reduce the accumulation of hydrophobic matrix material on the columns upon repeated injection of DBS samples. As a consequence of this observation, 2-propanol was employed as organic washing solvent and added to MP B, which was used for the analytical separation. The washing procedure was complemented by the introduction of water–acetic acid (100:1, v/v) as aqueous washing solution. In addition, the introduction of methanol–water (50:50, v/v) as extraction solution prevented the tubing between the clamp adapter and the 10-port valve from clogging with matrix components extracted from the DBS.

As first pre column, a LiChrospher 100 RP18 ADS (25 mm × 2 mm, 25 μm, Merck) was used for online sample preparation because the restricted access material is well suited for the direct injection of untreated biological fluids. The bimodal chromatographic properties of the column material allow retention of hydrophobic low-molecular weight analytes by RP chromatography at the hydrophobic pore surface, whereas macromolecules of the blood matrix are size-excluded by 6 nm pores.

For a more efficient sample cleanup prior to the analytical separation, a second pre column (CC 8/4 Nucleosil 100-5 C18 HD, 5 μm, kept at 50 °C; Macherey-Nagel), exhibiting a different selectivity compared to the restricted area material of the first pre column, was integrated into the LC system setup. This enabled further separation of matrix components resulting in significantly less pronounced ion suppression of analytes during MS detection compared to a one pre column setup. To minimize unnecessary loading of pre column 2 with blood matrix components that could impair analytical separation and MS/MS detection, both pre columns were switched inline only during the specific time window of analyte elution from pre column 1. Outside this period the LC flow from pre column 1 was directed into waste 2.

The differential temperature setting of pre column 2 (operated at 50 °C) and the analytical column (kept at 30 °C) allowed the initial re-focusing of analytes on the analytical column after their elution from the second pre column. This was due to less pronounced hydrophobic interactions with the RP column matrix at elevated temperatures [35]. Thus, by elevating the temperature under reversed-phase conditions, it is possible to significantly reduce the content of organic solvent while keeping the same eluent strength.

Furthermore, comparison experiments with large clamp adapters (inner diameter: 4 mm) and small clamp adapters (inner diameter: 2 mm) revealed that the adapter size did not have a significant impact on the results of the analysis in terms of the analyte signal-to-noise ratios. In fact, clamp adapters with an inner diameter of 2 mm (equivalent to 1.5 μL of blood) yielded similar results in terms of sensitivity compared to 4 mm clamp adapters. This is because the extraction of a larger area of a DBS did not only result in higher amounts of desorbed analytes but also in increased quantities of desorbed blood matrix components responsible for ionization suppression and noise level during MS/MS detection. Moreover, contamination of the mass spectrometer was considerably reduced with smaller adapters installed in the clamp module. As a consequence 2 mm adapters were used for the performance of the validation experiments.

The chemically treated DMPK-A cards and untreated DMPK-C cards (plain cellulose) gave similar analyte responses during method development. However, with DMPK-A cards slightly better analyte peak shapes were achieved. Therefore, the more expensive DMPK-A cards were chosen for further use in the validation. Due to moisture expansion of treated DMPK-B card material during analysis, which resulted in leaking, this card type was considered not suitable for SCAP DBS online extraction.

The temporal overlap of the flushing and re-conditioning phase of the LC system with the analytical separation and MS/MS detection, realized by synchronized valve switching, yielded a total cycle time of 9 min per analytical run. The apparently prolonged cycle time was necessary to achieve reproducible data for all four analytes. Bosentan and Ro 47-8634 exhibited a strong hydrophobic behavior during chromatography, while Ro 48-5033 and Ro 64-1056 showed less pronounced hydrophobic interactions. Therefore, the washing and transfer phases as well as the analytical gradient had to be adjusted accordingly to allow a quantitative transfer of analytes within the LC system and to achieve robust chromatographic conditions.

As a result of method optimization more than 500 repeated human DBS sample analyses could eventually be performed without a significant column backpressure increase or a decrease in chromatographic performance and sensitivity. The data obtained were highly reproducible, precise, and accurate.

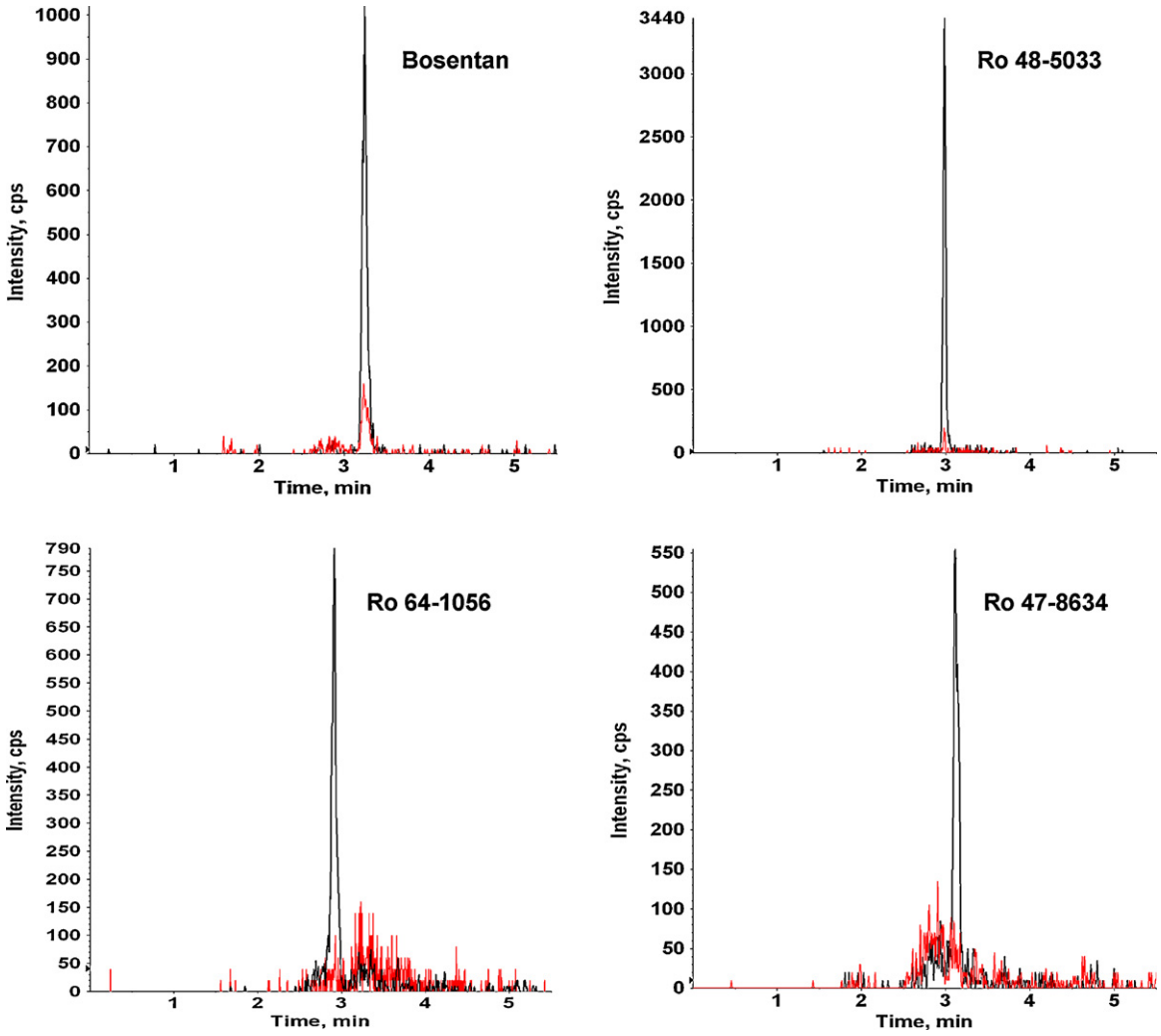

Fig. 2. Selectivity of the analytical method for bosentan, Ro 48-5033, Ro 64-1056, and Ro 47-8634. LC–MS/MS extracted ion chromatograms of bosentan, Ro 48-5033, Ro 64-1056, and Ro 47-8634 in human DBS blank sample (red trace) and human DBS blank sample spiked with all analytes at the respective LLOQ level (black trace). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Method validation

3.2.1. Whole blood stability

The analyte concentrations in human EDTA blood after storage for 6 h at ambient temperature showed a decrease of 0.3% at the QC_{low} level and a decrease of 14.4% at the QC_{high} level for bosentan, a decrease of 4.9% at the QC_{low} level and a decrease of 5.6% at the QC_{high} level for Ro 48-5033, a decrease of 5.0% at the QC_{low} level and an increase of 3.2% at the QC_{high} level for Ro 64-1056, and a decrease of 0.2% at the QC_{low} level and a decrease of 3.2% at the QC_{high} level for Ro 47-8634.

The analyte concentrations in human blood after storage for 2 days at $6 \pm 4\,^{\circ}C$ showed a decrease of 2.2% at the QC_{low} level and a decrease of 11.2% at the QC_{high} level for bosentan, a decrease of 5.6% at the QC_{low} level and a decrease of 4.0% at the QC_{high} level for Ro 48-5033, a decrease of 3.4% at the QC_{low} level and an increase of 5.6% at the QC_{high} level for Ro 64-1056, and an increase of 1.0% at the QC_{low} level and an increase of 1.6% at the QC_{high} level for Ro 47-8634.

This demonstrates that bosentan and its metabolites were stable in human blood for at least 6 h at ambient temperature and for at least 2 days at $6 \pm 4\,^{\circ}C$. Nevertheless it needs to be mentioned that with the whole blood stability testing procedure described here a potential degradation of analytes during the drying process cannot be investigated. This gap could however be bridged with a stability experiment conducted on liquid whole blood without spotting onto paper cards/without the use of the SCAP DBS System.

3.2.2. Linearity and sensitivity

Based on a sample volume of 25 μL the standard curves ranged from the LLOQ of 2 ng/mL (5 ng/mL for Ro 47-8634) up to 1500 ng/mL. The calculated correlation coefficients (r^2) for the three validation runs for accuracy and precision were ≥0.9957 for bosentan, ≥0.9954 for Ro 48-5033, ≥0.9970 for Ro 64-1056, and ≥0.9972 for Ro 47-8634.

The r^2 values determined for the calibration curves used for the other validation tests were ≥0.9957 for bosentan, ≥0.9951 for Ro 48-5033, ≥0.9969 for Ro 64-1056, and ≥0.9962 for Ro 47-8634. The deviation of the calculated standard concentrations from their nominal values was ≤15%, including the respective LLOQs.

3.2.3. Selectivity

During selectivity testing no significant interfering peaks were detected in the retention time windows of the analytes. The mean interferences in the DBS blank samples were 13.8% of the mean peak area of bosentan at the LLOQ level, 5.4% of Ro 48-5033, 5.2% of Ro 64-1056, and 5.1% of Ro 47-8634. The selectivity of the method was

Table 2
Inter- and intra-day accuracy and precision of the respective quality control samples for bosentan, Ro 47-8634, Ro 48-5033, and Ro 64-1056 in human DBS.

	Nominal concentration (ng/mL)	Mean of measured concentration (ng/mL)	Accuracy (%)[a]	Precision (CV%)[b]
Bosentan				
Intra-day[c] ($n = 6$)	2	2.09	104.6	10.5
	6	5.95	99.2	4.0
	120	123	102.4	3.3
	1250	1260	100.8	2.7
Inter-day[d] ($n = 18$)	2	1.89	94.7	14.6
	6	5.58	93.0	8.2
	120	123	102.5	4.6
	1250	1280	102.4	4.2
Ro 47-8634				
Intra-day[c] ($n = 6$)	5	4.30	86.0	14.8
	15	13.90	92.5	6.9
	120	117	97.6	2.4
	1250	1290	103.2	2.8
Inter-day[d] ($n = 18$)	5	4.58	91.6	13.1
	15	15.3	102.2	10.0
	120	126	104.9	7.1
	1250	1360	108.0	5.4
Ro 48-5033				
Intra-day[c] ($n = 6$)	2	1.99	99.7	6.1
	6	6.23	103.8	4.7
	120	127	105.9	3.5
	1250	1190	95.2	2.5
Inter-day[d] ($n = 18$)	2	1.88	94.2	7.1
	6	5.92	98.7	5.2
	120	127	106.0	4.7
	1250	1220	97.6	3.4
Ro 64-1056				
Intra-day[c] ($n = 6$)	2	2.07	103.7	10.9
	6	6.14	102.3	8.0
	120	126	105.3	3.5
	1250	1280	102.4	2.0
Inter-day[d] ($n = 18$)	2	2.01	100.7	10.3
	6	5.96	99.3	6.5
	120	126	105.0	4.9
	1250	1350	108.0	5.5

[a] Accuracy % = [(mean or overall mean concentration)/nominal concentration] × 100.
[b] CV% = (standard deviation/mean or overall mean concentration) × 100.
[c] Data obtained in first validation run.
[d] Data obtained in three validation runs.

therefore ensured. Fig. 2 shows the corresponding overlay chromatograms for each analyte.

3.2.4. Specificity

The evaluation of the specificity of the method yielded acceptable results for accuracy and precision. The accuracy was 102.9% for bosentan, 100.1% for Ro 48-5033, 102.5% for Ro 64-1056, and 92.4% for Ro 47-8634. The precision was 11.4% for bosentan, 14.7% for Ro 48-5033, 9.8% for Ro 64-1056, and 11.0% for Ro 47-8634. The method was therefore considered specific for bosentan and its metabolites.

3.2.5. Accuracy and precision

The method exhibited good accuracy and precision. The intra- and inter-assay accuracy was within 20% of the actual value at the LLOQ and within 15% at all other QC concentration levels. The precision determined at each concentration level did not exceed 15% of the CV (20% at the LLOQ level). No significant analyte responses were detected in the DBS blank samples. Table 2 summarizes the intra-day accuracy and precision data obtained in the first validation run during accuracy and precision testing and the inter-day accuracy and precision data from three separate days.

3.2.6. Matrix effect

The matrix effect is defined as the suppression or enhancement of the analyte ionization due to the presence of, e.g., endogenous matrix material such as peptides or phospholipids, metabolites, or components originating from plastic tubes used during sample preparation. Especially in ESI the matrix effect can be very pronounced [36]. The comparison of mean peak responses obtained from DBS blank samples with those obtained in the analysis of blank DMPK-A cards signified no substantial matrix effect for bosentan and Ro 48-5033 as well as their ISTDs (MF value close to 1). For Ro 64-1056 and its ISTD an MF greater than 1 was determined, suggesting ionization enhancement in the presence of blood matrix. In the case of Ro 47-8634 and its ISTD ion suppression was observed (MF < 1). Because of the similar chemical properties and elution times of the ISTDs relative to the analytes, the MFs for the analytes and their corresponding ISTDs are very similar. Therefore, the ISTD-normalized MFs for the analytes are close to unity.

The matrix effect arose from co-extracted material from the dried blood itself. Paper-based matrix effects originate from chemicals with which the DMPK-A cards are impregnated. These tend to be polar and as such introduce a region of matrix effects in the early eluting window of RP-chromatography [37]. Since the observed matrix effect was comparable for the ISTDs, accuracy and precision of the assay were not compromised. The ISTDs compensated for potential inconsistent responses due to matrix effects. The CV% from replicate measurements was ≤15%. The results of the matrix effect evaluation are shown in Table 3.

Table 3
Matrix factor and internal standard-normalized matrix factor for bosentan, Ro 47-863, Ro 48-5033, and Ro 64-1056 as well as matrix factor for the internal standards in human DBS.

Concentration level ($n = 6$)	Matrix factor[a]/ISTD-normalized matrix factor[b]			
	Bosentan	Ro 47-8634	Ro 48-5033	Ro 64-1056
QC_{low} [c]	1.05/0.96	0.56/0.90	0.98/0.95	1.53/1.09
QC_{high} [d]	0.85/0.78	0.51/0.82	0.85/0.83	1.31/0.94
Concentration level ($n = 6$)	Matrix factor[a]/ISTD-normalized matrix factor[b]			
	d4-Bosentan	d4-Ro 47-8634	d4-Ro 48-5033	d4-Ro 64-1056
QC_{reg} [e]	1.09	0.62	1.03	1.40

[a] Matrix factor: MF = Peak response$_{DBS\ blank\ sample}$/Peak response$_{Blank\ DMPK\text{-}A\ card}$.
[b] ISTD-normalized matrix factor: $MF_{norm} = MF_{analyte}/MF_{ISTD}$.
[c] QC_{low} corresponds to 6 ng/mL; 15 ng/mL for Ro 47-8634.
[d] QC_{high} corresponds to 1250 ng/mL.
[e] QC_{reg} corresponds to ISTD concentrations used in regular DBS analysis.

3.2.7. Recovery

In general, the extraction efficiency is determined by comparing the responses of DBS spiked with the analyte(s) at different concentrations with those of non-extracted standard(s) (reference values). For the automated online approach described here the recovery testing procedure was modified in order to take into account the automated platform of the assay. Hence, the response of a single extraction cycle versus the sum of responses of three consecutive extractions of the same spot at the identical position was evaluated. The DBS card was not released from the clamp module between the analyses. The recovery was considered as the online extraction efficiency of the system. As can be seen in Table 4, the recoveries for bosentan and its metabolites were consistent and ranged from 83.0% to 100%. The intra-assay precision was ≤2.1%. The data indicate a high recovery for all analytes and a very good precision.

3.2.8. Effect of hematocrit

Human DBS samples at the QC_{low} and QC_{high} level with hematocrit values of 0.35 and 0.65 gave acceptable results within ±15% of the measured mean concentration of QC samples with hematocrit of 0.45 (Table 5). From this it can be concluded that a hematocrit between 0.35 and 0.65 had no apparent effect on the quantitative determination of analyte concentrations in human DBS samples. The CV% from replicate measurements was between 1.6 and 9.0%.

Despite the fact that no assay bias with different hematocrits was observed the result and its consequences need to be critically examined. Human blood hematocrit influences the spread of blood on DBS filter paper cards, the formation and the size of the DBS and eventually robustness and reproducibility of the assay. This is particularly the case when a subsample from the DBS is taken. At a high hematocrit, the distribution of a fixed volume of blood sample through the filter paper might be poor, resulting in a smaller blood spot when compared with the blood sample with a low hematocrit. Since the volume of blood within the fixed diameter subsample (extraction area) of the DBS also increases it would be expected that the bias in measured analyte concentrations would increase with increasing percentage hematocrit. On the other hand, the extraction efficiency of analytes may decrease with increasing hematocrit even for compounds that have low affinity for blood cells, such as bosentan and its metabolites. As a result of these counteracting effects the bias obtained from varying hematocrits would be (partially) masked making it appear as if there is no significant influence of hematocrit on the quantitative determination of analytes in DBS samples.

To conclusively elucidate possible counteracting effects for bosentan and its metabolites the analyte extraction efficiency at different hematocrits would need to be investigated.

3.2.9. Carryover

The experiments revealed a slight sample carryover for bosentan and its metabolites following the extraction of the highest calibration standard (ULOQ). The mean carryover for the analytes, determined from triplicate measurements, was 0.26% for bosentan, 0.15% for Ro 48-5033, 0.19% for Ro 64-1056, and 0.28% for Ro 47-8634. The mean carryover extent in the second blank DBS sample following the ULOQ sample injection was shown to be well below the corresponding LLOQ levels. With regard to the integrity of validation data the observed carryover was not critical. The carryover did not significantly affect the accuracy and precision of the assay. To exclude the possibility that the determination of analyte concentration levels in study samples is affected by the carryover (false-too-high concentration data), blank samples should be introduced after samples with an expected high concentration, before the analysis of the next study sample. Furthermore, randomization of samples should be avoided.

3.2.10. Robustness/influence of blood spot volume

The QC sample results obtained with increasing blood volumes were within ±15% of the nominal values (Table 6). From this it can be concluded that a spotted blood volume between 20 and 30 μL had no significant influence on measured analyte concentrations. Precise sample pipetting may not be necessary.

Table 4
Recovery for bosentan, Ro 47-8634, Ro 48-5033, and Ro 64-105 in human DBS, three consecutive extractions (entire run cycles) of the same DBS area.

Concentration level ($n = 6$)	Recovery (%)			
	Bosentan	Ro 47-8634	Ro 48-5033	Ro 64-1056
QC_{low} [a]	83.0	100	100	100
QC_{mid} [b]	92.3	98.0	96.3	94.6
QC_{high} [c]	92.3	98.0	94.4	94.3

[a] QC_{low} corresponds to 6 ng/mL; 15 ng/mL for Ro 47-8634.
[b] QC_{mid} corresponds to 120 ng/mL.
[c] QC_{high} corresponds to 1250 ng/mL.

Table 5
Hematocrit assessment for bosentan, Ro 47-8634, Ro 48-5033, and Ro 64-1056 in human DBS (0.35, 0.45, and 0.65 hematocrit). Calibration curve prepared at hematocrit 0.45.

Nominal concentration (ng/mL) ($n=6$)	Hematocrit					
	0.35		0.45		0.65	
	Precision (CV%)[a]	Deviation to hematocrit 0.45 (%)[b]	Precision (CV%)[a]	Deviation to hematocrit 0.45 (%)[b]	Precision (CV%)[a]	Deviation to hematocrit 0.45 (%)[b]
Bosentan						
6	7.3	+1.5	6.9	0.0	7.9	+5.9
1250	3.7	+1.0	6.5	0.0	3.4	+9.1
Ro 47-8634						
15	5.0	−8.9	4.0	0.0	4.5	+2.5
1250	4.4	+2.4	3.5	0.0	2.8	+12.6
Ro 48-5033						
6	5.8	+1.1	5.9	0.0	3.0	+0.3
1250	7.7	+2.8	1.6	0.0	3.4	+10.4
Ro 64-1056						
6	6.4	−5.0	4.2	0.0	9.0	+3.4
1250	2.3	+0.2	3.1	0.0	5.1	+8.7

[a] CV% = (standard deviation/mean concentration at respective hematocrit) × 100.
[b] Deviation% = ((mean concentration at respective hematocrit/mean concentration at hematocrit 0.45) × 100) − 100.

Table 6
Influence of blood spot volume on the measured concentrations of bosentan, Ro 47-8634, Ro 48-5033, and Ro-64 1056 in human DBS. Samples were measured along with a set of calibration standards, for which the spotted blood volume was 25 μL.

Nominal concentration (ng/mL) ($n=6$)	Blood spot volume (μL)					
	20		25		30	
	Mean (ng/mL)	Accuracy (%)	Mean (ng/mL)	Accuracy (%)	Mean (ng/mL)	Accuracy (%)
Bosentan						
6	5.70	94.9	5.87	97.8	5.68	94.7
1250	1280	102.4	1350	108.0	1290	103.2
Ro 47-8634						
15	14.9	99.4	16.6	110.5	16.4	109.1
1250	1390	111.2	1430	114.4	1400	112.0
Ro 48-5033						
6	5.94	99.0	6.17	102.8	6.01	100.2
1250	1290	103.2	1350	108.0	1300	104.0
Ro 64-1056						
6	5.87	97.8	6.15	102.5	6.18	103.1
1250	1380	110.4	1430	114.4	1400	112.0

3.2.11. Long-term stability of DBS

It could be shown that bosentan and its metabolites were stable in DBS for at least 3.5 months at ambient temperature (Table 7). The individual deviations from the reference values were within ±15% for each analyte. The precision was between 1.8% and 12.8%.

Table 7
Stability of bosentan, Ro 47-8634, Ro 48-5033, and Ro 64-1056 in human DBS over 3.5 months at ambient temperature.

Concentration level ($n=6$)	Precision (CV%)	Deviation to t_0 (%)[a]
Bosentan		
QC_{low} [b]	9.1	−5.6
QC_{high} [c]	1.8	−6.6
Ro 47-8634		
QC_{low} [b]	12.8	−8.6
QC_{high} [c]	2.4	−12.4
Ro 48-5033		
QC_{low} [b]	3.8	+0.3
QC_{high} [c]	2.6	−11.5
Ro 64-1056		
QC_{low} [b]	2.8	−6.2
QC_{high} [c]	2.5	−14.0

[a] Deviation% = ((mean concentration at t = 3.5 months/mean concentration at t_0) × 100) − 100.
[b] QC_{low} corresponds to 6 ng/mL; 15 ng/mL for Ro 47-8634.
[c] QC_{high} corresponds to 1250 ng/mL.

3.2.12. Stock solution stability

Stock solution stability of the analytes could be demonstrated for 5 months storage at 6 ± 4 °C. The intra-assay precision was ≤6.9%, and the deviation between the mean areas of the samples made from stored and freshly prepared stock solutions was ≤3.0% for all analytes (data not shown).

4. Conclusions

The SCAP DBS System allows the fully automated online bioanalysis of pharmaceutical compounds in DBS samples. The method validation for the quantification of bosentan and its three primary metabolites in human DBS was successfully conducted following the FDA guidance for bioanalytical method validation [31]. The good sensitivity of the assay is reflected by the fact that the DBS area of extraction was only 4 mm^2, which is equivalent to 1.5 μL of blood.

The SCAP DBS technology opens up new ways in DBS bioanalysis and provides a robust and promising tool to the pharmaceutical industry and the clinical sector. The degree of automation obtained with the SCAP DBS System obviates the need for tedious punching of DBS samples and also for subsequent offline extraction and centrifugation steps. This makes sample throughput (time per sample that is required for its preparation and analysis) competitive with

that obtained in conventional liquid sample analysis, i.e., parallel processing in the 96-well format.

Compared with manual extraction in direct DBS analysis relatively dirty extracts may be introduced into the LC-MS system. The experiments performed during method validation clearly revealed the importance of implementing extensive washing phases in online DBS analysis. This is to prevent accumulation of blood matrix material such as phospholipids and proteins in the analytical system and thus to ensure robust and reproducible performance over hundreds of consecutive DBS samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.12.012.

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