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On-line desorption of dried blood spot: A novel approach for the direct LC/MS analysis of μ-whole blood samples

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ABSTRACT

The aim of this work is to present a new concept, called on-line desorption of dried blood spots (on-line DBS), allowing the direct analysis of a dried blood spot coupled to liquid chromatography mass spectrometry device (LC/MS). The system is based on an inox cell which can receive a blood sample (10 μL) previously spotted on a filter paper. The cell is then integrated into LC/MS system where the analytes are desorbed out of the paper towards a column switching system ensuring the purification and separation of the compounds before their detection on a single quadrupole MS coupled to atmospheric pressure chemical ionisation (APCI) source. The described procedure implies that no pretreatment is necessary in spite the analysis is based on whole blood sample.

To ensure the applicability of the concept, saquinavir, imipramine, and verapamil were chosen. Despite the use of a small sampling volume and a single quadrupole detector, on-line DBS allowed the analyses of these three compounds over their therapeutic concentrations from 50 to 500 ng/mL for imipramine and verapamil and from 100 to 1000 ng/mL for saquinavir. Moreover, the method showed good repeatability with relative standard deviation (RSD) lower than 15% based on two levels of concentration (low and high). Function responses were found to be linear over the therapeutic concentration for each compound and were used to determine the concentrations of real patient samples for saquinavir. Comparison of the founded values with those of a validated method used routinely in a reference laboratory showed a good correlation between the two methods. Moreover, good selectivity was observed ensuring that no endogenous or chemical components interfered with the quantitation of the analytes.

This work demonstrates the feasibility and applicability of the on-line DBS procedure for bioanalysis.

1. Introduction

Since 1960, the filter paper 903 S&S produced by Whatman has been widely used as support for collecting newborns' capillary blood for the screening of neonatal metabolic disorders [1–4].

Called dried blood spots (DBS), this procedure offers the advantage of being less invasive and more cost effective in terms of sample collection, shipment and storage as compared to venipuncture. These advantages are now well established by different publications [5–7]. The actual trends in biomedical analysis are to reduce biological material, time and analysis costs. Recent advancements and developments in analytical technology, especially in mass spectrometry, have allowed greater improvements in terms of sensitivity and selectivity [8]. Taking advantage of that, the dried blood spots sampling has been recently applied to the monitoring of pharmaceutical compounds including antimalarials [9,10], antiepileptics [11], antiretrovirals [12,13], antibiotics [14,15], and immunosuppressive drugs [16,17].

However, one weakness of the procedure is the off-line extraction step; compounds need to be extracted from the filter paper before analysis. To our knowledge, the extraction procedure is usually carried out by an organic solvent or a mixture of water–methanol, containing an internal standard. After extraction and centrifugation, the supernatant is collected and analysed by LC/MS. In comparison, on-line DBS, which integrates already the advantages of the DBS procedure, allows without any pretreatment, the direct analysis of a small whole blood sample (10 μL) coupled to a conventional column switching LC/MS system which is known to provide easier and faster sample preparation by combining extraction and analysis step into one integral process [18].

Saquinavir (SAQ) is one of the HIV protease inhibitors (PI) used in the highly active antiretroviral therapy (HAART) in association with two nucleoside reverse transcriptase inhibitors (NRTI). Owing to the high risk of patient non-compliance due to unwanted side effects

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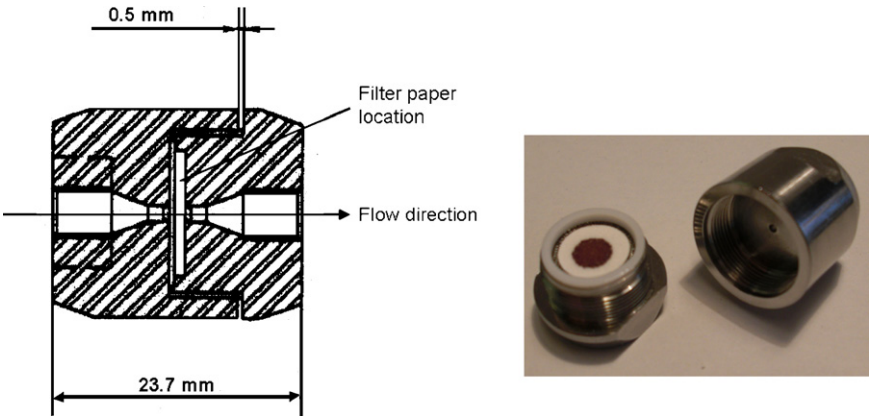

Fig. 1. Schema and picture of the desorption cell used for on-line DBS process.

and the number of tablets per day, therapeutic drug monitoring of saquinavir is highly recommended to control patient compliance and to avoid viremia increasing [19].

Imipramine (IMI) is a tricyclic antidepressant prescribed since 1980. Its pharmacological action is attributed with increasing the synaptic concentration of the noradrenergic and serotonergic neurotransmitters. Due to the numerous side effects that may lead to patient's death, therapeutic drug monitoring (TDM) of IMI is recognized as level 1 (highly recommended) by the TDM expert group consensus guidelines [20].

Verapamil (VER) is a selective calcium entry inhibitor in cardiac muscle and systemic arteries. Thus, it is employed for its antiarrhythmic, antianginal and antihypertensive properties. Furthermore, VER is one of the most effective inhibitors of P-glycoprotein (Pgp) [21,22], widely used in different models to describe the role of Pgp on the availability of pharmaceutical compounds.

This work presents a novel technique which integrates, for the first time, a DBS filter paper with LC/MS system. The procedure is particularly interesting since no pretreatment is needed although whole blood is introduced directly into the LC/MS system.

2. Experimental

2.1. Chemicals and solutions

SAQ and saquinavir D_9 (SAQ D_9) were obtained from Roche discovery Welwyn (Welwyn Garden City, UK). VER, IMI and trimipramine D_3 (TRI D_3) were purchased from Cambridge Isotope Laboratory (Andover, USA).

Working standard solutions were prepared by dilution of stock solutions in methanol to reach concentrations of interest ranging from 1 to 10 μg/mL, excepted for SAQ D_9 and TRI D_3, used as internal standard (IS), which were prepared by dilution of stock solutions in acetonitrile (ACN) to reach concentrations of 1 μg/mL and 500 ng/mL, respectively. After use, stock and working solutions were stored at −20 °C.

Both ACN and methanol (MeOH) were of high-performance chromatographic grade from Merck (Darmstadt, Germany). Formic acid (FA) was also obtained from Merck (Darmstadt, Germany). Human blood was supplied by the Hôpitaux Universitaires Genevois (Geneva, Switzerland).

2.2. DBS sample processing

10 μL of real or spiked whole blood sample was spotted on a filter paper card, item no. 10535097 from Whatman, Schleicher and Schuell (Dassel, Germany). The blood spots were allowed to dry at room temperature for 30 min and then packed in a sealable plastic bag containing silica gel. Each case was analyzed after a 24-hour waiting period. A 10-mm diameter disc containing DBS was punched out of the card and 5 μL of IS solution was added directly on the DBS. Finally, the blood disc was placed into the desorption cell as described in Section 2.3.

2.3. System description

The system is based on an inox cell in which a piece of filter paper, containing the blood spot, can be placed. Fig. 1 presents the schema and a picture of desorption cell geometry. This one was designed and built by ourselves to be compatible with LC capillaries. Sealing is ensured by a ring in fluorinated plastic (external–internal diameters of 14 and 12 mm, respectively, and thickness of 1.5 mm). The internal diameter of the desorption cell was machined to receive 12 mm diameter blood spots (corresponding to a 50 μL DBS) [7].

The desorption cell was then introduced into the LC system (Agilent series 1100 system, Waldbronn, Germany) as depicted in Fig. 2, to form the on-line DBS concept. To ensure automation of the procedure, a system of electro-valves was employed. Thus, a 6-port valve (Valve 1) was used to connect the desorption cell to the column switching system [23], using extractive (Chromolith Flash RP-18e, 25 mm × 4.6 mm) and analytical (Chromolith Performance RP-18e, 100 mm × 4.6 mm) columns linked together by a second 6-port valve (Valve 2). A 12-port valve allows semi-automation since 6 supports can be placed on-line by switching to the next position after the end of each run. This configuration represents the first approach towards the development of an automate allowing the full on-line desorption of DBS.

In this configuration, Pump 1 (100% ACN) was used to desorb and elute the analytes from the filter paper towards the extractive column with a flow rate of 0.25 mL/min. Pump 2 (100% H_2O, 0.1% FA) was fixed at 2.25 mL/min to adjust the chemical properties of the desorption mobile phase by the use of a tee connector. Thus the global flow rate eluting through the extractive column was maintained at 2.5 mL/min. Pump 3 delivered the analytical mobile phase constituted by a mixture of ACN (A) and water (B) both with 0.1% FA at 1.5 mL/min.

After extraction time (t = 5.5 min), Valve 2 was switched to position 1 to connect the extractive and analytical columns. The trapped analytes are then desorbed in the back flush mode and transferred to the top of the analytical column by the analytical mobile phase of 2 min duration. Then, Valve 2 was returned to its initial position 0, allowing the precolumn to be regenerated. Finally, a generic gradient was used to ensure the wide separation of pharmaceutical compounds in an acceptable run time. Table 1 describes the instrumental setting for the on-line DBS LC/MS procedure.

Table 1
Instrument setting for the on-line DBS LC/MS procedure. Pump 1 works with 100% ACN, Pump 2 works with 100% H_2O 0.1% FA and Pump 3 delivers mobile phase with a constant flow rate at 1.5 mL/min.

Time (min)	Valve position		Pump 1	Pump 2	Pump 3	
	V1	V2	Flow (mL/min)	Flow (mL/min)	% ACN 0.1% FA	% H_2O 0.1% FA
0.00	0	0	0.00	0.00	20	80
0.01	1	0	0.25	0.00	20	80
0.49	1	0	0.25	0.00	20	80
0.50	1	0	0.25	2.25	20	80
5.00	0	0	0.25	2.25	20	80
5.01	0	0	0.00	2.25	20	80
5.50	0	1	0.00	2.25	20	80
7.50	0	0	0.00	2.25	20	80
14.50	0	0	0.00	2.25	90	10
14.51	0	0	0.00	0.00	90	10
16.00	0	0	0.00	0.00	90	10
16.10	0	0	0.00	0.00	20	80
18.00	0	0	0.00	0.00	20	80

Detection of the compounds was carried out by a single quadrupole mass spectrometer Agilent series 1100 MSD single quadrupole VL version, equipped with an APCI interface (Agilent, Waldbronn, Germany). The MS was operated in positive ion mode. The following instrumental settings were used: drying gas (N_2) flow of 5 L/min, drying gas temperature of 325 °C, nebulizer pressure of 60 p.s.i, capillary voltage of 4000 V, vaporizer temperature of 400 °C, Corona current of 4 μA. The fragmentor voltage (skimmer) was 80 V. The dwell times were placed at 174 ms for each compound.

2.4. Analytical experiment

On-line DBS concept was first tested by carrying out the accuracy and repeatability of the method. For each chosen compound, a response function was realized over the therapeutic range by 5 levels of calibration from 50 to 500 ng/mL for IMI and VER and from 100 to 1000 ng/mL for SAQ with 2 repetitions per level. Two quality controls were prepared independently in sextuple ($n = 6$) at two levels of concentration corresponding to the lower and higher levels of therapeutic range of the drugs.

To test the selectivity of the method, several bloods ($n = 6$) were spotted on filter paper and then analysed using on-line DBS procedure to show if there were interferences between the endogenous compounds and the tested drugs. Matrix effects were also determined by post-infusion column to determine if on-line DBS leads to either ion suppression or enhancement phenomena.

Finally, a comparison was carried out between on-line DBS approach and a reference LC-MS/MS method [24] to determine the correlation between the two methods.

3. Results and discussion

3.1. Desorption of the filter paper

3.1.1. Desorption solvent

In on-line DBS configuration, the issue was to find the optimal conditions to ensure a selective desorption of the interest analytes compared to the matrix interferences.

Generally, DBS desorption is based on a mixture of water and an organic solvent such as MeOH or ACN. Fig. 3 presents the result obtained in on-line DBS configuration after both water and ACN desorption. Water seems to be more efficient since a complete desorption of the blood spot was observed compared to ACN for which the spot was similar before and after desorption. However, the comparison of the MS signal response obtained for each compound showed that the difference between the two desorption solvents was no significant. Moreover, the use of water leaded to contamination of the ionisation source generating a rapid degradation of the MS signal (data not shown), which did not happen when ACN was used.

These observations are confirmed by the obtained UV–vis spectra of the desorption flow. Indeed, the one after water desorption is characteristic of the UV–vis blood spectrum with bands at 350,

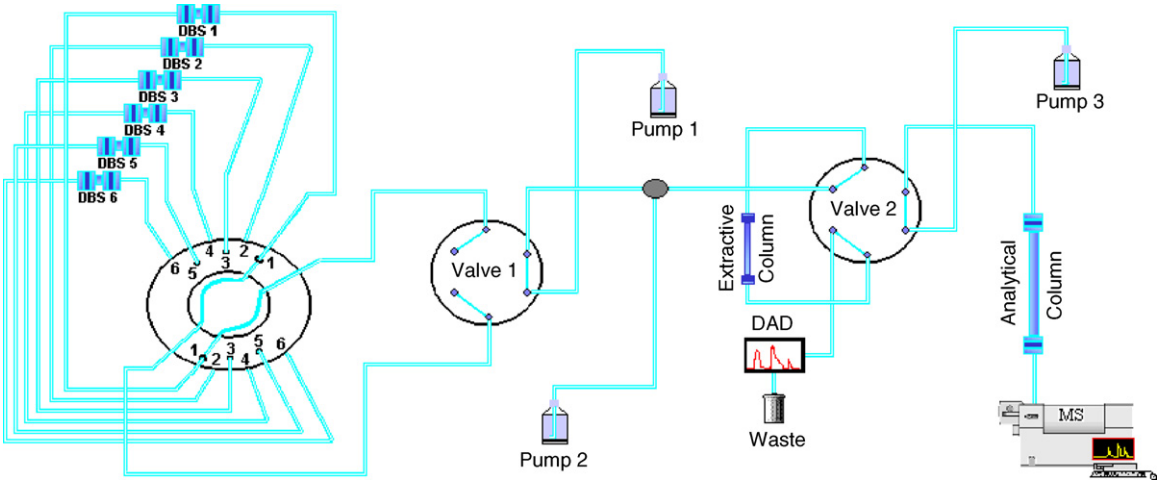

Fig. 2. Global view of the on-line DBS procedure coupled to column switching LC/MS system.

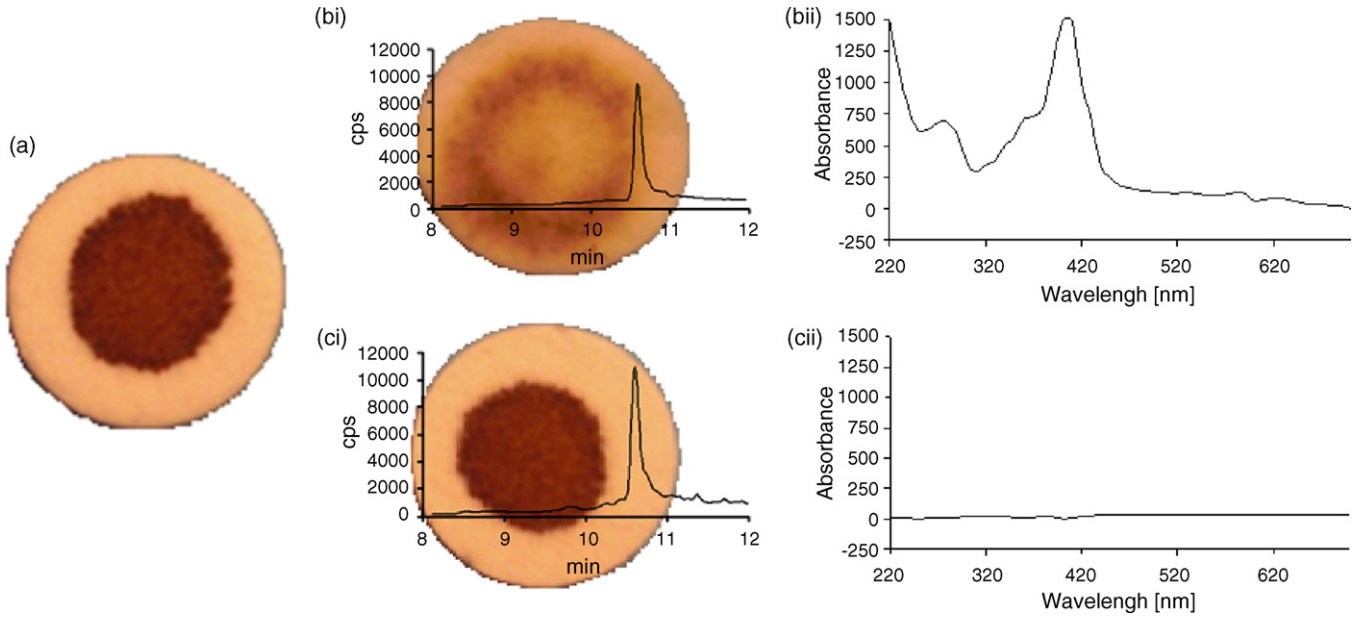

Fig. 3. Pictures and associated chromatograms in SIM mode of on-line DBS containing verapamil at 125 ng/mL (i) and associated UV–vis spectra of desorption flow (ii), before desorption (a), after H_2O desorption (b), and ACN desorption (c).

412, 576, and 620 nm due to the absorption of iron porphyrin of haemoglobin and a band at 280 nm due to the protein absorption [25]. Inversely, the one after ACN desorption shows that no protein or more particularly haemoglobin are desorbed from the filter paper that explain why the filter paper have the same visual aspect before and after desorption. Furthermore, the use of other organic solvents like methanol, isopropanol, and acetone has given the same results than ACN (data not shown).

Compared to water desorption, organic solvent desorption seems to lead to a precipitation of the proteins which agglomerate into the paper fibres. In this way, proteins such as haemoglobin are not eluted into the desorption flow.

3.1.2. Trapping on the extractive column

Although ACN is a solvent of choice for on-line desorption, it does not permit an efficient trapping of the analytes in the reverse phase extractive column. Based on these considerations, the problem was bypassed by the use of a tee which allowed adjusting polarity of the mobile phase by adding a higher flow of water. Thus, in front of the extractive column, the percent of the ACN, which was 100% during the desorption step, was reduced to a suitable ratio for a good trapping (usually lower than 10%).

3.1.3. Desorption flow

Study of the desorption was performed, with dried blood spots of verapamil, at different concentrations ranging from 0.5 to 10 μg/mL and using different flow rates ranging from 0.25 to 1.00 mL/min. As expected, the results have shown that the desorption time is flow rate dependent. However, desorption flow rate delivered by Pump 1 is limited by the fact that Pump 2 has to work with higher water flow to dilute the ACN before trapping (see Section 3.1.2). Therefore, the choice of the Pump 1 flow needs to be a compromise between desorption effectiveness and Pump 2 limitations. Hence, Pump 1 was fixed at 0.25 mL/min for 5 min which seems to be the best compromise for our application to obtain a satisfying desorption with an acceptable delay (see Fig. 4). Moreover, these settings imply to maintain Pump 2 (100% water) at a flow rate of 2.25 mL/min leading to 10% of ACN in the extractive mobile phase. The global flow rate of the extractive mobile phase then was of 2.5 mL/min which is in agreement with the use of monolithic column. It is obvious that desorption time could be decreased according to the requested sensitivity and the detector used.

Another aspect to take into account is the efficiency of the desorption according to the spotted amount of compounds. For a desorption flow rate fixed at 0.25 mL/min, Fig. 4 shows that the observed response was directly proportional to the amount of verapamil spotted on filter paper over the concentration range what implies that the desorption efficiency stays the same regardless of concentration.

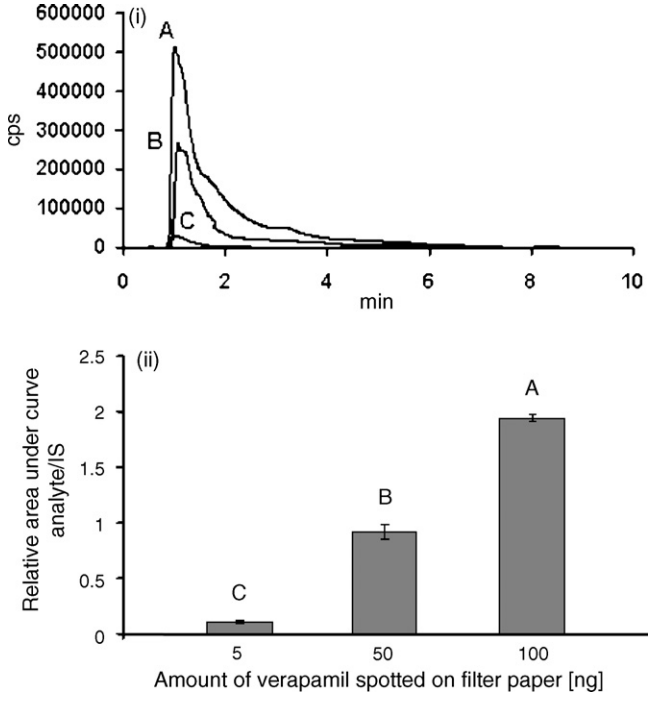

Fig. 4. Selected ion chromatograms (i) of 10 μL dried blood spots containing verapamil at 10 μg/mL (A), 5 μg/mL (B) and 0.5 μg/mL (C) desorbed at 0.25 mL/min and related graph of the efficiency desorption after three repetitions by concentration level (ii).

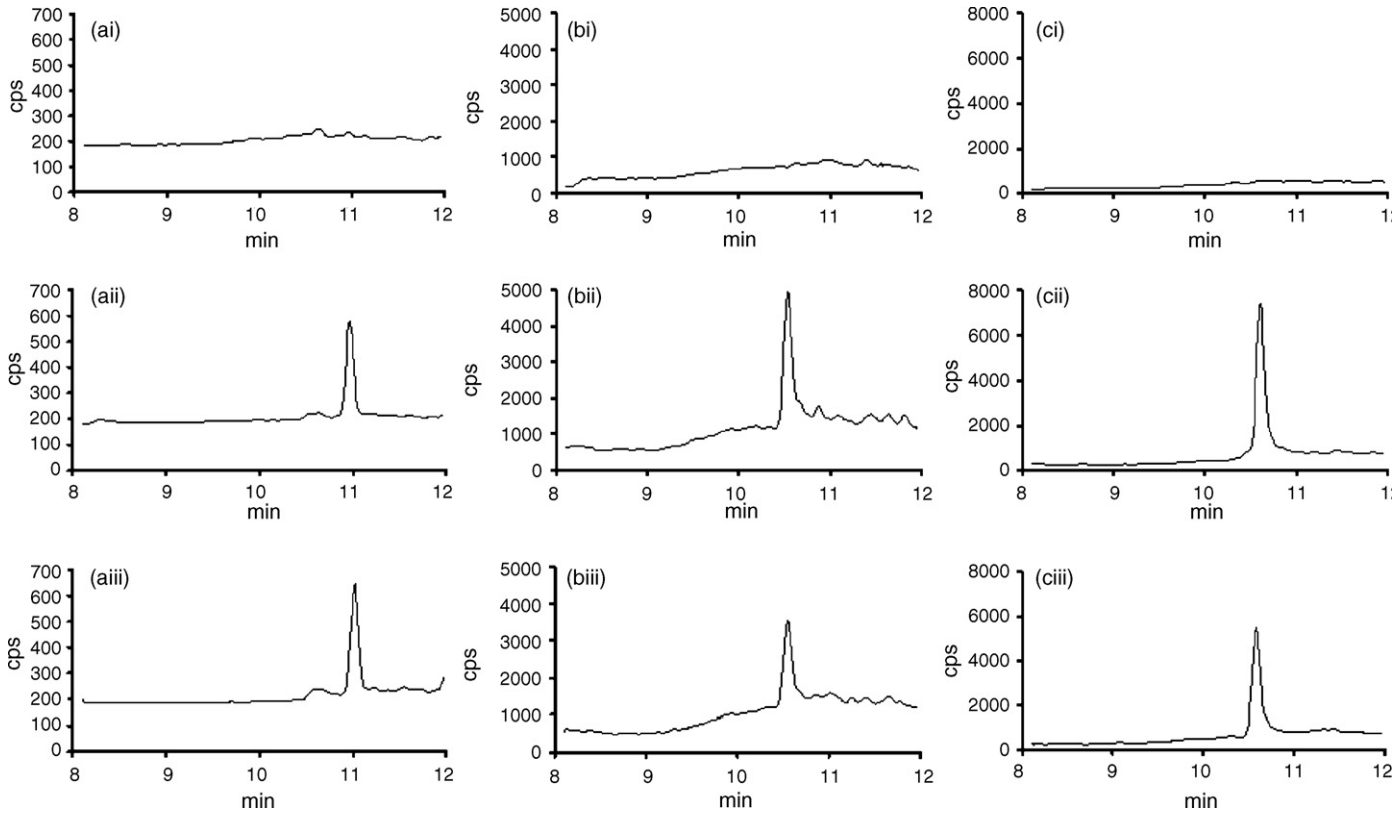

Fig. 5. Representative selected ion monitoring chromatograms of DBS for saquinavir (a), imipramine (b), and verapamil (c) with a blank DBS spiked with internal standards (i), a DBS patient sample closed to lower level of therapeutic range (ii), and a quality control DBS at lower level of therapeutic range (iii).

3.2. Performance of the system

On-line DBS process allows the quantitative analysis of 10 μL of whole blood, previously spotted on filter paper, with a single quadrupole as detector acquiring in SIM mode. To evaluate the performance of the system, determination of accuracy and precision was carried out. Table 2 shows the quantification performance data obtained for the three compounds simultaneously. Accuracy was included between 90.5 and 116.1% for the three compounds and relative standard deviations (RSDs) were lower than 15%.

Although a single quadrupole and 10 μL of blood were used, good sensitivity was observed for the compounds allowing their quantitation over their therapeutic range (see Fig. 5). Another advantage is that each analysis uses a new support leading to a decrease in the carry-over phenomena known to be problematic in conventional LC systems where autosampler is used. Carry-over with on-line DBS process was investigated by injecting a blank solution after the injection of two times the highest calibration point. The results show that no carry-over is observed with our procedure (lower than 0.01%).

Table 2
Quantification performance data obtained for the three compounds.

Criteria	Saquinavir	Imipramine	Verapamil
Accuracy (%)			
50 ng/mL	–	97.7	116.1
100 ng/mL	105.2	–	–
500 ng/mL	–	90.8	104.0
1000 ng/mL	90.5	–	–
Precision (%)			
50 ng/mL	–	13.8	6.8
100 ng/mL	8.4	–	–
500 ng/mL	–	8.1	13.9
1000 ng/mL	8.9	–	–

3.3. Matrix effect and selectivity

Due to the direct desorption of whole blood into LC/MS system, eventual matrix interferences need to be investigated even if APCI is known to be less sensitive to matrix effects than electrospray ionisation (ESI). Indeed, several examples of both ion suppression and enhancement have been reported when APCI is used [26–28]. For these reasons, evaluation of the matrix effect was performed by post-column infusion experiments consisting of the analysis of DBS placed in desorption cell while the analytes were infused in the mobile phase after the analytical column [29]. As shown in Fig. 6, any variation of the MS response was observed implying that our system does not present enhancement or suppression effect at the retention time window of the compounds.

Selectivity was also treated by spotting different whole blood samples ($n = 6$). The results showed that no interference peaks were co-eluted at the retention time of the selected compounds.

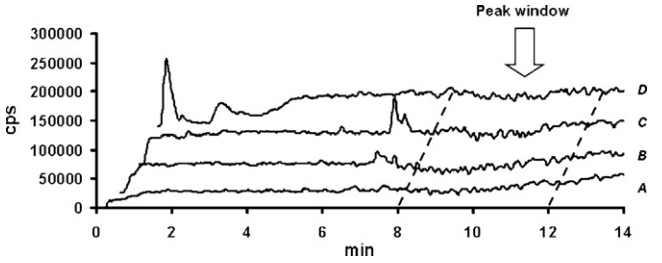

Fig. 6. Matrix effect investigation of a blank filter paper (A) and 3 different whole blood spotted on filter paper (B–D) performed by post-column infusion in positive SIM detection mode.

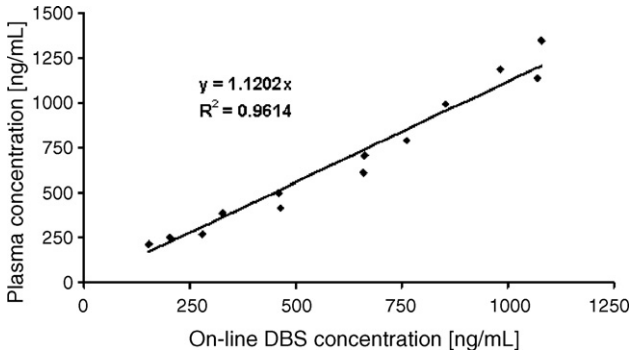

Fig. 7. Comparison of saquinavir levels in patient samples ($n = 13$) between on-line DBS and validated LC-MS/MS using plasma.

3.4. Comparison of on-line DBS versus validated LC–MS/MS procedure on patient samples

The described procedure was then compared to a reference LC-MS/MS procedure developed for the routine analysis of SAQ [24]. As described above, 10 μL of whole blood samples was spotted on the filter paper before the rest of each sample was heated at 60 °C during 30 min and centrifuged at the Clinical Pharmacology Division of Centre Hospitalier Universitaire Vaudois (Lausanne, Switzerland) where the analyses were carried out on the plasma. Fig. 7 shows the results obtained for the comparison of the two procedures on 13 patient samples. We found here a good correlation between the analyses carried out in plasma samples versus on-line DBS samples, since the obtained slope was 1.12 with a satisfying correlation coefficient which is in agreement with other published data [12].

4. Conclusion

For several decades, DBS sampling has been used in diverse fields of application as neonatal hereditary disorders screening, TDM, screening of biomarkers [30], pharmacokinetics, and pharmacodynamics studies (DMPK, ADME) [31,32].

This interest for this sampling procedure can be explained by its minimally invasive property, compared to venipuncture, which is particularly suitable for sensible patients such as newborns with limited available quantity of blood. Furthermore, DBS sampling is appropriated to decrease contact with infectious material, and to facilitate samples mailing and storage. However an off-line extraction of the analytes from DBS is often required before their analysis by LC or immunoassay.

The procedure described in this article has permitted, for the first time, the on-line integration of filter paper into column switching LC/MS system. Thus, the advantages of the DBS sampling are now coupled with a no sample pretreatment procedure.

The most important goal in the future is to focus on the development of high throughput on-line DBS methods to be more convenient with pharmaceutical or clinical attempts. In this way, the first approach is to use a more sensitive MS detector to decrease the desorption step and therefore the total run time. The second approach, which is in development in our laboratory, is the conception of an autosampler where multiple filter paper disks will be set simultaneously allowing the complete automation of the described concept.

Although a single quadrupole and 10 μL of blood were used, on-line DBS was successfully applied to the analysis of interest drugs in TDM or pharmacokinetic approach. This novel procedure will constitute a powerful solution for bioanalytical analysis in the future.

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