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Approach To Bioanalytical Method Development by Dry Blood Spot and LC-MS/MS Using Saquinavir Drug as An Analyte

KEYWORDS: Dry Blood Spot, Saquinavir, LC-MS/MS, Pharmacokinetics, Toxicokinetics, Therapeutic drug monitoring, Bioanalysis

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ABSTRACT *Dry blood spot (DBS) is a micro sampling technique to collect blood samples on a suitable paper to predominantly screen medical disorders by analyzing the dried blood spots using different analytical techniques. In the past few years DBS has gained significance in drug development due to its ethical and logistical advantages over conventional sampling in pharmacokinetic (PK), toxicokinetic(TK) and therapeutic drug monitoring (TDM) studies. Bioanalysis of DBS samples obtained from PK, TK and TDM studies require sensitive and reproducible bioanalytical method to accurately measure the concentration of drugs in these samples. DBS method development is quite challenging, especially for low sensitivity assays. A comprehensive approach was adopted that resulted in a sensitive and a reproducible DBS-LC-MS/MS method for a therapeutic drug. The reported method provides an insight into DBS method development;same approach can be useful for a wide range of drugs of similar nature.*

Saquinavir drug was used as an analyte to develop a DBS bioanalytical method by LC-MS/MS quantitation. Sample preparation parameters related to DBS card selection, sample spotting, sample drying,sample punching, suitability of extraction solvent and sample mixing were evaluated thoroughly. Agilent DMS card gave optimal results than other DBS cards tested during the optimization (for example: DMPK-A, B and C and Tomtec-200, 300and 400).The method demonstrated 2000 fold linearity from 5.056 ng/mL to 10062.500 ng/mL. The mean recovery of Saquinavir was 41.6%. The overall precision (% CV) and accuracy (% nominal) of the method was within ± 15% and 85.0% to 115.0% respectively. The method can be applied to routine bioanalysis for Saquinavir.

INTRODUCTION

Dry blood spot (DBS), a micro sampling technique,is a blood sample collection technique used in diagnostics, drug discovery and drug development applications [1].DBS is relatively a simpler technique with multiple advantages over conventional sampling technique. The DBS is less invasive (typically 0.1mL or less in DBS sample collection by finger or heel prick) is a major advantage over conventional sampling (typically 0.5mL in conventional sample collection by venipuncture). It also promotes ethical use of animals for pharmacokinetic (PK) and toxicokinetic (TK) studies [2]. The ease of sample handling, storage and transportation due to dried format nature of DBS samples is additional advantage.

The major breakthrough in DBS came in 1957when Robert Guthrie first collected the blood samples of a new born on a filter paper to measure phenylalanine to evaluate metabolic disorders [3].

DBS micro sampling technique is also useful for DNA-based assay, chromatography analysis, immunoassays and enzyme activity assays[4].DBS technique is used in several clinical applications for neonatal screening, microbiological and epidemiological disease surveillance, therapeutic drug monitoring (TDM), clinical pharmacology and clinical pharmacogenetics [4].Generally serum and plasma are the common matrices for routine TDM in patient samples but collecting and handling of such samples at study sites issometimes quite challenging and results in non-compliance and inconclusive outcome. DBS-LC-MS/MS has been increasingly employed in clinical studies and TDM for analysis of a wide spectrum of drug molecules, including cardiovascular therapy [5], immunosuppressant therapy [6, 7, 8, 9], psychoactive drugs [10], antibiotics [11], vinca alkaloids [12]and in monitoring drug abuse[13, 14].

In the last few years, due to the simplicity of DBS technique and its multiple advantages it has been applied for bioanalysis to measure drug concentrations in the blood samples collected from PK,TK and TDM studies.

The significant difference between DBS and conventional blood sample collection is differentiated by quantitation of drug in whole blood versus plasma. The DBS technique can be applied for range of nonclinical and clinical studies provided DBS method is accurate and precise to measure drug concentrations in blood with desired sensitivity.

DBS method development poses quite few challenges for low sensitive assays but a systematic approach adopted for sample preparation and use of a sensitive analytical technique play a crucial role to establish a sensitive, reliable and robust method .

Saquinavir chosen as an analyte for DBS method development is an antiretroviral drug used in HIV therapy and it belongs to a class of protease inhibitors. The protease inhibitors prevent viral replication by selectively binding to viral proteases. Saquinavir is used in combination with other antiretroviral to delay the progression of HIV infection. The clinical outcome of various protease inhibitor combinations is vital for research on HIV drugs requires rigorous clinical compliance for accuracy of results and desired clinical outcome. DBS sample collection from HIV patients enrolled for clinical trials can improve clinical compliance and intended study outcome. The samples collected by DBS technique need accurate and reproducible DBS bioanalytical methodology to analyse PK samples to draw desired conclusions.

The typical Saquinavir human dose ranges from 200-1000 mg in combination with other HIV drugs. The reported-Cmax concentration of Saquinavir varies in the range of about 500-7000 ng/mL [15, 16, 17, and 18].In order to accurately measure the drug concentration profile in the blood from absorption to elimination phase at all dosing ranges, the desired sensitivity required for quantitation of Saquinavir in human PK samples is about 5-10 ng/mL.Some of the methods were reported for quantitation of Saquinivir in serum with sensitivity of 10ng/mL [19], in plasma with sensitivity of 7.8ng/mL [20]and 27.3ng/mL [21, 22],by using LC-MS/MS method. Few methods were reported in literature for quantitation of Saquinavir by using DBS and LC-MS/MS method with the sensitivity of 50 ng/mL [23] and 25 ng/mL [24] as LLOQ.

The DBS method of Saquinavir described in this research paper is unique in terms of its methodology and sensitivity at 5.056ng/mL can be applied to bioanalysis of DBS samples obtained from low to high dose range of PK, TK and TDM studies.

EXPERIMENTAL

Chemicals and Reagents

Saquinavir Mesylate (Lot GSMC0440001, Purity: 98.0%) and Doxepin (Lot 117K0683, Purity: 100%),Figure-1 were obtained from Toronto Research ChemicalsInc. Canada and Sigma Aldrich respectively. HPLC grade acetonitrile and methanol were obtained from Merck. Formic acid was supplied by Sigma Aldrich. Milli-Q water with 18.2 MΩ/cm resistivity was obtained from in-house Milli-Q water purification system, Millipore, USA. The dry blood spot (DBS) cards tested during the sample preparation were DMPK cards A, B and C of GE and Agilent DMS card of Agilent, USA. The blank rat whole blood was obtained from in-house animal facility of Nektar Therapeutics,Hyderabad in accordance with the guidelines related to animal ethics.

Analytical instrumentation

A Shimadzu SILHTc – 20 AD (Shimadzu Corporation, Japan) consisting of flow control valve, column oven, degasser and auto sample operated in gradient mode to deliver the mobile phase. The chromatographic system consists of reverse phase C_{18} column ACE 75mm × 4.6 mm, 5µ internal diameter (Grace-Vydac USA).

API-4000 mass spectrometry with Electron Spray Ionization (ESI) and multiplereaction monitoring (MRM) mode was used as a detector for data acquisition (AB Sciex, USA). Peak integration and calibration were carried out by inbuilt Analyst 1.5.1 software.

Both ESI positive and negative ion modes were tested with different mobile phase combinations like methanol, acetonitrile and water/ammonium acetate buffer (1-3mM)/formic acid (0.1-0.2%). MS and MS/MS condition for Saquinavir(analyte) and internal standard (ISTD)Doxepin were optimized by continuous infusion using syringe pump at10 µl/min. Them/z of 671.5/570.3and 671.5/416.1 transition for analyte and 280.4/106.9 transitions for ISTD were monitored. The analysis was carried out in ESI positive ion mode with spray voltage set at 5500 V. The electro spray ionization source temperature was set 450°C. The curtain gas,GS1, GS2 and collision associated dissociation gas (CAD)were set at25, 30, 40 and 10 psi respectively. The optimized compound dependent parameters are presented in Table 1.

Mobile phase consisted of water (mobile phase A)and acetonitrile (mobile phase B) containing 0.1% (v/v) formic acid. Isocratic mobile phase condition was used for total run time of 3.5 minutes with 50:50 of mobile phase A and mobile phase B. The auto sampler and column temperature were set at 5°C and 45°C respectively and the 10 µL injection volume was used during analysis.

Preparation of stock, calibration standards and quality control samples

The stock solutions of Saquinavir (0.67 mg/mL) and Doxepin(1.00 mg/mL) were prepared in methanol: water (80:20 v/v) mixture and stored at 2-8°C. 1000.00ng/mL of Doxepin was prepared in methanol: water (80:20 v/v) mixture and used as a working internal standard solution (WISTD). The working solutions of calibration standards and quality control samples of Saquinavir were prepared by serial dilution of the stock solutions in methanol: water (80:20 v/v). The calibration (range: 5.056 to 10062.500ng/mL) and quality control(QC) standards (14.490, 4830.000, 6900.000ng/mL as low, mid and high respectively) were prepared by spiking analyte working solution in blank SpragueDawley (SD) rat blood and then spotted on DBS cards.

DBS card selection

Different DBS cards namelyDMPK series A, B, C, Tomtec series 200, 300, 400 and Agilent DMS were tested for suitability of sample spotting, sample drying and sample punching. 15, 20, 30 and 40µL sample volumes were spiked on the card to check sample holding capacity and drying interval of the card. 3 mm spot punching, full spot punching and full circle punching was done to check the effect of sample spread and extent of recovery of analyte from the spots. Multiple spotting of samples was done on same card to check cross talk of samples due to surface tension. Impact on recovery of analyte in freshly collected blood and stored blood was evaluated. Absolute recovery was also tested.

For card selection, sample spotting was done by spiking 5 µL of 5000 ng/mL working solution of analyte in 95µL freshly collected rat blood. Contents were mixed gently. 15 µL of spiked blood was spotted on each DBS card. All sample cards were allowed to dry overnight at ambient temperature. Dried blood spots were punched neatly by a manual puncher and transferred to 2 mL eppendorf tubes. 400 µl of 0.1% Formic acid in acetonitrile was added to extract analyte from the dry sample. The contents were vortexed and centrifuged. The supernatant was injected on LC-MS/MS. Based on visual inspection and comparative peak areas of analyzed samples of all cards,Agilent DMS card was found most suitable for method development.

Optimization of sample preparation
Based on consistency in recovery, Agilent DMS card was used to further optimize sensitivity and reproducibility of the method.The procedures were tested in a stepwise manner to optimize sample volume for spotting, solvent combinations for extraction of analyte from DBS sample, solvent volume and mixing conditions to achieve best possible sensitivity and reproducibility. To optimize recovery, different extraction solvents tested were 0.2% formic acid water, 0.1%formic acid acetonitrile and combinations of solvents like 10%water 90%acetonitrile acidified with 1% formic acid, 20% water 80%acetonitrile acidified with 0.1% formic acid, 30%water 70% acetonitrile acidified with 0.1% formic acid, 20% water 80% methanol acidified with 0.1 % and 0.2% formic acid, 15% water 85% methanol acidified with 0.2% formic acid. The solventvolumes tested during extraction were 100, 200, 400 and 1000 µL. The vortex mixing was evaluatedfor 1, 3, 5, 10, 60 minutes. Injection volume was 2 and 10 µL. Mixing conditions were altered to test the impact on analyte recovery.

Based onthe conclusions of different tests performed as mentioned above, the sample preparation that gave the best performance was selected by spiking 5 µl of analyte working solution in freshly collected 95 µl of SD rat whole blood and gently mixing the contents. 15 µl of this sample was spotted on Agilent DMS card. The card was allowed to dry overnight at ambient temperature in a vacuum desiccator containing activated silica gel. The full dried spot samples were cut neatly and transferred into a 2mL eppendorf centrifuge tube.400 µl of extraction solvent containing 15% water 85% methanol v/vacidified with 0.2% formic acid was added along with15 µl of 1000ng/mL of Doxepin as ISTD. The sample contents were vortexed for 10 minutes and centrifuged at 14000 rpm for 5 minutes. The supernatant was transferred to auto sampler vial and 10 µl sample injected into chromatographic system for detection by AB sciexAPI-4000 mass spectrometer.

Evaluation of method robustness
The final method involving DBS sample preparation, chromatographic separation and mass spectrometer detection(DBS-LC/MS-MS) were adopted to assess the linearity, precision and accuracy,extraction efficiency (recovery), matrix effect and bench top stability to evaluate the method robustness and method suitability for bioanalysis of routine samples.

LOQ determination
The LOQ was determined based on analyte response by spotting four samples with LOQ concentration of analyte at 5.056ng/mL and four blank samples on DBS cards. The samples were analyzed to compare with response of LOQ with blank samples.

Linearity
The standard calibration curves were constructed using the peak area ratio of Saquinavir and Doxepin versus Saquinavir nominal concentrations in whole blood(calibration standards concentrations were5.056, 10.113, 50.564, 505.641, 1011.281, 2022.563, 4045.125, 6037.500, 8050.000, 10062.500ng/mL). Quadratic regression with weighting factor $1/x^2$ was performed to assess the linearity. In addition, a blank (whole blood spotted as blank on DBS) and a zero blank sample (whole blood spotted on DBS and ISTD added during sample preparation) were run to demonstrate the absence of interferences at analyte and ISTD retention time respectively.

Precision and Accuracy
Precision and accuracy of the method was evaluated using three different batches of quality control sample at four different concentrations at 5.056,14.490, 4830.000, 6900.000 ng/mL.Each batch was quantified with a specific calibration curve. For each intra-batch assay precision (%CV) and accuracy evaluation, four replicates of quality control samples at four concentration levels were used. The inter-batch assay precision and accuracy were determined from three analyzed batches to obtain the corresponding inter-batch precision and accuracy.

Extraction Efficiency (Recovery)
The recovery experiment was performed at three QC levels (Low QC, Mid QC and High QC at 14.490, 4830.000, 6900.000ng/mL respectively) with three replicates at each concentration. The extraction efficiency was evaluated by calculating the area ratio at each level. The mean area ratio of extracted samples compared with unextracted(aqueous sample) samples at each respective QC levels.

Matrix effect
Matrix effect was evaluated at two QC levels (Low QC and High QC at 14.603 and 6953.884ng/mL respectively) with four replicates at each level using extracted and unextracted samples. The extracted samples were prepared by extracting blank DBS spots with extraction solvent. The samples were dried and QC solutions were spiked in the dried samples along with extraction solvent. The analyte response was compared with unextracted samples prepared same way without blank DBS spots.

Bench top stability
The bench top stability was evaluated at ambient temperature by spotting two QC levels on DBS card (Low QC and High QC at 14.603 and 6953.884ng/mLrespectively) with four replicates at each level. The samples were dried and stored in a desiccator till analysis. The stability comparison was done with freshly spotted samples.

RESULTS AND DISCUSSION

Mass spectrometryand Chromatography
The positive electro spray ionization (ESI) mode was adopted for or the quantification of analyte. The signal intensity of analyte and ISTD in ESI positive ion mode was 2-3 fold higher than the ESI negative ion mode. The protonated molecular ion of $[M + H]^+$ of the analyte and ISTD was observed at m/z= 671.53and 280.4Da respectively. There was no solvent adduct ions or fragment ions observed in the full scan spectra. On the basis of these observations, positive ion mode was selected for detection and quantization of analyte, which on fragmentation gave prominent and stable product ions. The details are presented in Table-1

Good chromatography in terms of peak width and shape was obtained with optimized isocratic mobile phase of acidified acetonitrile and water rather other buffers. A reverse phase C_{18} column ACE 75mm×4.6mm, 5µ gave acceptable results with total analysis run time of 3.5 min. The retention time of analyte and ISTD was about 2.0 minutes and 1.5 minutes respectively. Representative chromatograms of extracted blank, LLOQ and ISTD (Doxepin) samples are presented inFigure 2, 3 and 4 respectively.

DBS card selection & sample preparation
15 to 30 µL sample was found suitable for spiking on Agilent DMS card. The vertical spiking of samples without pipette tip touching the card was most appropriate way to spike samples for accuracy and reproducibil-

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ity. The sample spread after spotting was uniform in all cards. The spread was relatively more in DMPK cards than Tomtec and Agilent DMS cards. The sample in DMPK-B card turned black while as no such change in colourwas observed in rest of the cards.

Among DMPK, Tomtec and Agilent DMS cards, the DMPK card B showed analyte recovery of 26% in 100% acetonitrile acidified with 0.1% formic acid and 18.5% recovery in 50% acetonitrile and 50% water combination acidified with 0.1% formic acid extraction solvents.Aqueous phase enhanced the analyte recovery from dried blood samples. All other cards showed<10% recovery under similar conditions. Analyte recovery enhanced from <10% to 32–44% in DMPK and Agilent DMS cards by using 90% acetonitrile 10% water acidified with 0.1% formic acid. However uneven spreading was noted in DMPK cards. The sample also got decolorized in DMPK card B and sample drying took relatively moretime. The analyte recovery was also inconsistent in DMPK cards in solvent combinations mentioned above. On the other hand,under similar conditions, the Agilent DMS card exhibited optimum performance with respect to spot sampling, even spreading of sample, drying, punching and recovery. The testing for card selection and sample spotting are shown in Figures 6, 7 and 8.Agilent DMS card did not exhibit significant variation in analyte response in whole blood samples with different hematocrit (HCT) values were tested.

The Agilent DMS card gave best recovery in the mixture of acidified methanol and water. 85% methanol and 15% water acidified with 0.2 % formic acidwas selected as solvent of extraction. Full circle punching using400 µL solvent mixture of was found adequate to extract Saquinavir from dried spot. The optimum vortexing time was 10 minute. No peak area variation was observed in the extracted samples which were kept at ambient temperature for 60 min suggesting no significant degradation upon storage.There was no improvement in recovery observedby equilibration of the dry spot sample in extraction solvent before or after mixing.

LOQ determination

The criteria to define the Limit of quantitation (LOQ) of Saquinavir were based on comparison between noise level or area of the analysed blank samples and the peak response of analyte in the spiked samples at analyte retention time. LOQ response was more than five times than noise level in the blank samples. The precision and accuracy of the analyte response at LOQ of 5.056ng/mL was <20%. The method is good enough to quantify the 5.056ng/mL as LOQ in 15 µL DBS blood spot.Sensitivity data is presented in Table-2 along with intra-assay precision and accuracy data.

This assay sensitivity (5.056 ng/mL) is greater than the reported methods in literature at 50 ng/mL [23] and 25 ng/mL as a LLOQ[24] using DBS. Some of the methods were reported for quantitation of Saquinavir in serum at 10ng/mL[19], and in plasma at 7.8ng/mL [20] and 27.3ng/mL [21, 22] as a LLOQ.

The DBS method reported is more sensitive than the plasma and serum as a matrix even with less volume of the blood sample.

Linearity

The peak area ratios of analytes to ISTD in SD rat blood were linear over the concentration range 5.056ng/mL to 10062.5 ng/mLfor Saquinavir. The calibration model was selected based on the analysis of the data by different regression models. The best fit for the calibration curve achieved by quadratic regression with $1/x^2$weighting factor. The correlation coefficients (r^2) was greater than 0.995. The representative curve is presented in Figure 5.

Precision and accuracy

The intra-day assay precision and accuracy for analyte wasevaluated by analysing six replicates at four different quality control(QC) levels. Inter-day assays precision was determined by analysing four levels QC samples on three different runs. The overall precision (% CV) and accuracy (% nominal) was within ± 15% and 85.0% to 115.0%respectively. The intra-assay precision and accuracy of one representative batch data is presented in Table -2.

Extraction efficiency (Recovery)

The extraction recovery was relatively low in DBS samples when compared to recovery of Saquinavir in plasma samples of reported studies. But recovery in DBS was consistent. The extraction efficiency was calculated between peak area of extracted and aqueous sample. Recovery (%) of analyte at LQC, MQC and HQC was 44.5 and 38.3 and 42.2 respectively. The mean recovery of quality control samples was 41.6%. The recovery data is presented in Table 3.

Matrix Effect

The analyte response of analysed extracted and unextracted samples did not exhibit any ion suppression or ion enhancement. The percent matrix effect was calculated from mean peak response of samples spiked after extraction divided by the mean peak response of neat samples times hundred (100). The mean of matrix factor for analyte and internal standard was 1.009 and 1.034 respectively. The mean normalized matrix factor of Low QC and High QC was 0.9766 with overall precision (%CV) of 2.7. The data is presented in Table 4

Bench Top Stability

The stability and comparison samples were analyzed using a freshly spotted DBS sample calibration curve. There was no significant degradation of analyte observed in stability samples when stored in desiccator at ambient temperature upto 12 days . The overall % change observed in Low QC and High QC stability samples was -13.5% and overall precision (%CV) of stability and comparison samples was 3.3%. The data is presented in Table 5.

CONCLUSIONS

The DBS method for Saquinavir described in this research paper,is unique in terms of sensitivity, provides a broad insight into method development approach by bio who are keen to explore DBS technique as an alternative tool for bioanalysis of drugs. Due to sensitivity and the robustness of the assay, this DBS-LC-MS/MS bioanalytical method of Saquinavir can be applied to a wide range of studies to quantify Saquinavir in whole blood of different species collected by DBS sampling technique in PK,TK and TDM studies.The selection of appropriate DBS card (Agilent DMS in this case) and extraction solvent (mixture of acidified methanol water combination in this case) were key considerations for achieving the sensitivity and reproducibility of the assay. Agilent DMS card was found most suitable for even spreading of blood spot, ease of spotting, minimal impact due to hematocrit variation, drying and punching. The full circle punching of the spot was found useful for enhancing sensitivity, accuracy and precision of the method.

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The approach adopted in selecting the method parameters resulting in anaccurate and precise method for Saquinavir can beapplied for method development and routine bio-analysis of similar drugs.

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Table 1: Compound dependent (MRM conditions) for quantitation of Saquinavir(Analyte) and Doxepin (ISTD)

Parameters	Saquinavir	ISTD
Q1	671.5	280.4
Q3	570.3, 416.1	106.9
Dwell Time	200	100
DP	100	65
CE	44, 53	37
CXP	21, 15	6
EP	10	10

Table 2: Intra-assay precision and accuracy of Saquinavir by using DBS and LC-MS/MS method

S. No.	LLOQ (5.056 ng/mL)		LQC (14.490 ng/mL)		MQC (4830.000 ng/mL)		HQC (6900.000 ng/mL)	
	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy
1	5.372	106.3	11.300	78.0	5264.211	109.0	7198.791	104.3
2	5.190	102.6	12.452	85.9	5226.598	108.2	6884.801	99.8
3	6.522	129.0	13.036	90.0	4505.506	93.3	7450.856	108.0
4	4.659	92.2	12.501	86.3	5457.703	113.0	7531.148	109.1
Mean	5.44	-	12.32	-	5113.50	-	7266.40	-
SD	0.78	-	0.73	-	417.79	-	291.15	-
% CV	14.4	-	5.9	-	8.2	-	4.0	-
% Nominal	107.5		85.0		105.9		105.3	

Table 3: Extraction efficiency (recovery) of Saquinavir by using DBS and LC-MS/MS method

S. No.	LQC		MQC		HQC	
	AQ	DBS	AQ	DBS	AQ	DBS
1	6487	2912	1923374	745025	2290339	990041
2	5881	2784	1897351	741557	2310985	991681
3	6547	2716	1889191	698060	2265862	913433
Mean	6305	2804	1903305	728214	2289062	965052
% Recovery	44.5		38.3		42.2	
Mean % Recovery	41.6					
% CV	7.5					

Table 4: Matrix effect of Saquinavir by using DBS and LC-MS/MS method

QCs (N=4)	Low QC 14.603 ng/mL			High QC6953.884ng/mL		
	Post extracted Peak area	Unextracted peak area	Analyte Matrix factor	Post extracted peak area	Unextracted peak area	Analyte Matrix factor
Mean	9559	9367	1.021	4472198	4487746	0.997
SD	127.70	232.21	0.01	134093.03	33963.07	0.03
%CV	1.3	2.5	1.3	3.0	0.8	3.0

Table 5: Bench top stability of Saquinavir by using DBS and LC-MS/MS method (12 days at ambient temperature)

Quality Control	LQC				HQC			
Nominal Conc. with Low and High Limit (ng/mL)	14.603				6953.884			
	12.413		16.793		5910.801		7996.967	
Samples	Stability		Comparison		Stability		Comparison	
S. No.	Cal. Conc.	% Accuracy	Cal. Conc.	% Accuracy	Cal. Conc.	% Accuracy	Cal. Conc.	% Accuracy
1	12.690	86.9	14.155	96.9	5400.813	77.7	6198.553	89.1
2	12.768	87.4	14.090	96.5	5719.131	82.2	6573.285	94.5
3	12.437	85.2	13.876	95.0	5520.757	79.4	6946.586	99.9
4	13.314	91.2	14.659	100.4	5365.986	77.2	6855.124	98.6
Mean	12.8023	NA	14.1950	NA	5501.6718	NA	6643.3870	NA
SD	0.36926		0.33150		159.41129		336.42917	
%CV	2.9		2.3		2.9		5.1	
% Nominal	87.7		97.2		79.1		95.5	
N	4		4		4		4	
% Change	-9.8				-17.2			

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Figure 1: Chemical Structure of Saquinavir (Analyte) and Doxepin (ISTD)

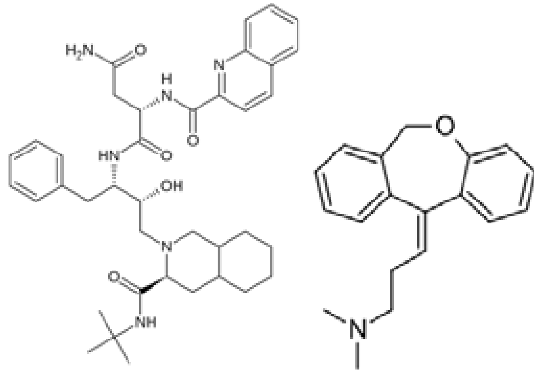


Figure 2: Representative chromatogram of Blank from DBS card

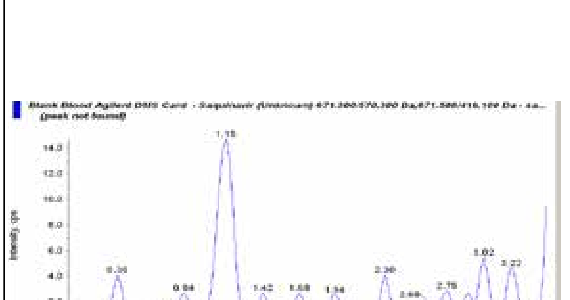


Figure 3: Representative chromatogram of LLOQ from DBS card

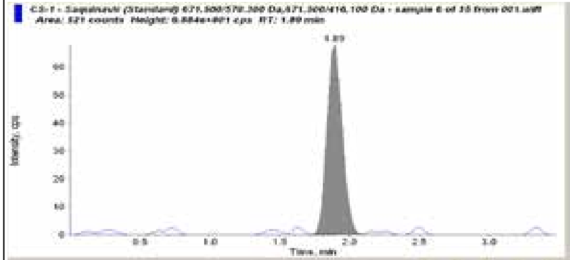


Figure 4: Representative chromatogram of Doxepin (ISTD) from DBS card

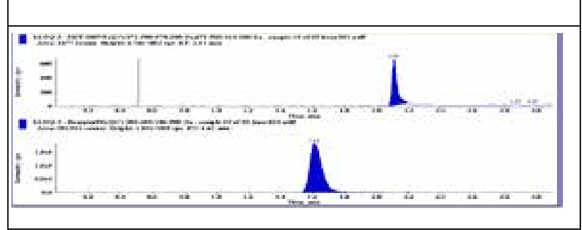


Figure 5: Representative calibration curve of Saquinavir

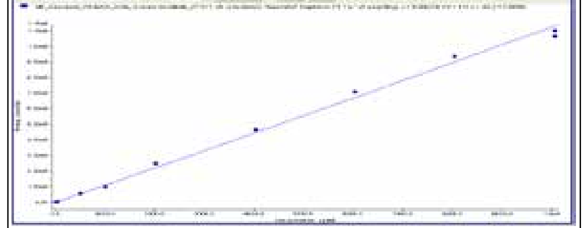


Figure 6 : Selection of DBS Card

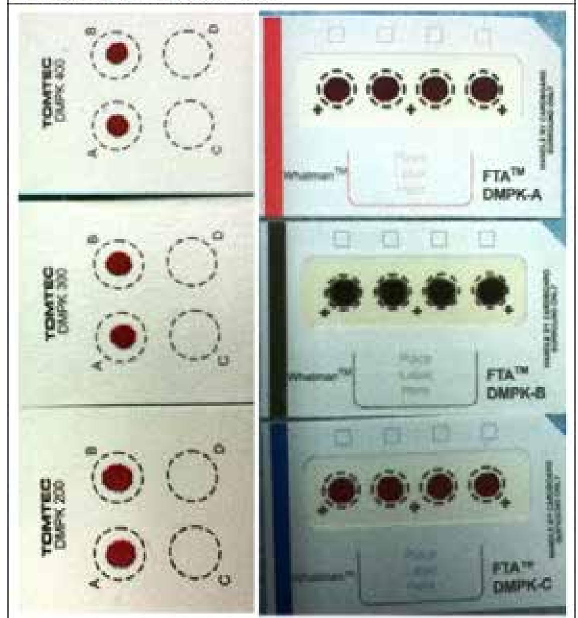


Figure 7: Spiking Technique and Assessment of Cross Talk of Samples due to Surface Tension

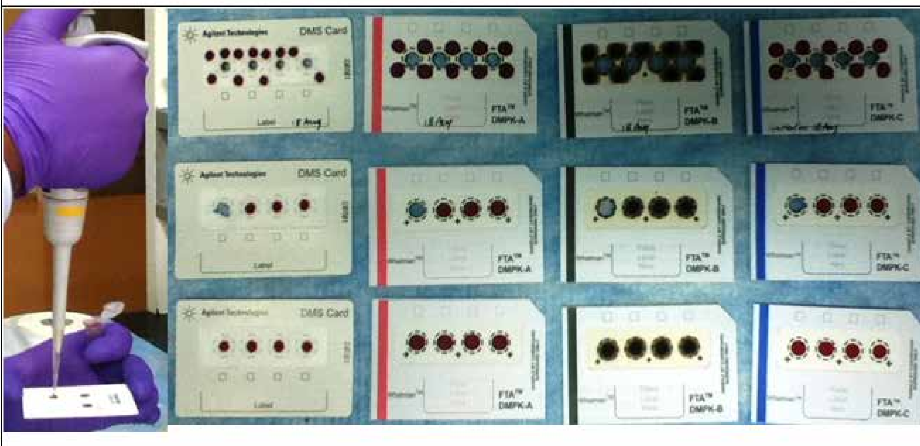
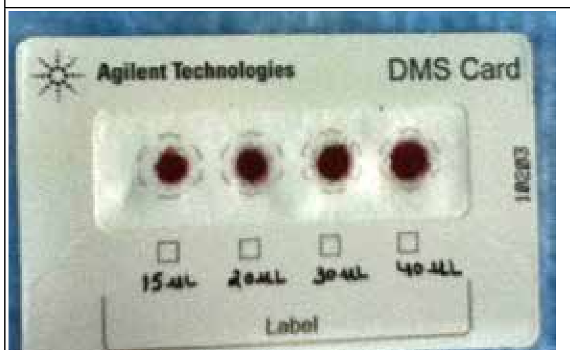


Figure 8 : Optimization of Spotting Volume



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