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ORIGINAL ARTICLE

Simultaneous Determination of Dofetilide and Amlodipine on Dried Blood Spot by using FLD: Application to Pharmacokinetics in Rat

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ABSTRACT
A simple, accurate, precise, sensitive and reliable high performance liquid chromatography with fluorescence detection (FLD) method was developed and validated for simultaneous determination of dofetilide (DFT) and amlodipine (AML) on dried blood spots (DBS). A dried blood spot technique was employed for rat blood collection followed by extraction of drug from DBS before analysis. The effective chromatographic separation was achieved on Kromasil C8 column (250 x 4.6 x 5 µm) using mobile phase containing mixture of 20 mM ammonium acetate pH 5.0 with acetic acid and acetonitrile in the ratio of 60:40, v/v in an isocratic elution mode at a flow rate of 1.0 mL min^{-1} with column oven temperature of 40 °C. The fluorescence detection were monitored at 240 and 370 nm as excitation and emission wavelength for DFT and 240 and 460 nm as excitation and emission wavelength for AML. The method was validated for accuracy, precision, linearity and selectivity. The robustness was performed by applying design of experiments (DoE) following ICH guidelines. The assay exhibited a linear range of 2–500 ng mL^{-1} for DFT and 5–1000 ng mL^{-1} for AML on dried blood spots. The lower limit of detection was found to be 0.7 and 1.2 ng mL^{-1} for DFT and AML respectively. The method exhibited good performance with intra- and inter-day precision %CV of 0.75 – 8.64 % and accuracy of 90.41 - 100.10% for both DFT and AML including all experimental levels. The method was successfully applied to a pharmacokinetic study in rats.

Keywords: *Liquid Chromatography, Dofetilide; Amlodipine; Fluorescence detection (FLD); Design of experiment (DoE); Dried blood spot (DBS); Pharmacokinetic study*

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INTRODUCTION

Cardiac arrhythmia is a condition in which there is irregular heartbeat, tachycardia is a condition in which heart rate is above 100 beats per min in adults and in bradycardia heart rate is below 60 beats per min. Dofetilide (DFT), chemically is *N*-[4-(2-{[2 (methane sulfonamide phenyl) ethyl] (methyl) amino} ethoxy) phenyl] methane sulfonamide as shown in Figure.1a [1-3]

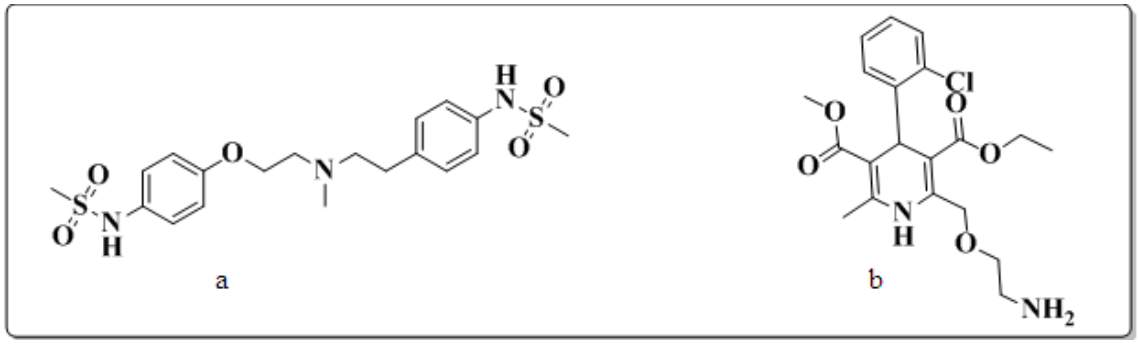

Figure. 1 Chemical Structure of (a) Dofetilide (DFT) and (b) Amlodipine (AML)

(TIKOSYN®)DFT is a Class III anti-arrhythmic drug developed for the treatment of reentrant tachycardia that selectively inhibits the rapid component of the delayed rectifier cardiac potassium current (IKr), which prolongs the action potential duration and the effective refractory period in a concentration dependent manner.[4-5]It acts by maintaining normal sinus rhythm in patients with atrial fibrillation, atrial flutter and supraventricular tachycardia.[6-8] It is highly potent drug and is effective at concentration in the nanomolar range. DFT prolongs the refractory period and action potential duration (APD) without having sodium channel-blocking effect, beta-blocking action or calcium channel-blocking activities. Excessive prolongation of APD, an extension of DFT pharmacological effect, results from an abnormal increase of cytosolic Ca2+ concentration which carries the risk of inducing early after depolarizations (EAD's). Amlodipine (AML), a calcium channel blocker, reduces the monophasic APD-prolonging effect of dofetilide suggesting a potential reduction of its proarrhythmic properties and it is the possible rationale for using both drugs simultaneously.[9-13]

Amlodipine besylate (AML), chemically is 3-ethyl- 5-methyl (±)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1, 4- dihydro-6-methyl- 3, 5- pyridine dicarboxylate monobenzenesulphonate, as shown in Figure.1b AML is a dihydropyridine calcium antagonist that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. The contractile processes of cardiac muscle and vascular smooth muscle are dependent upon the movement of extracellular calcium ions into these cells through specific ion channels. Amlodipine inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells.[14-15].

Keeping in mind the potential usefulness and safety, combined use of dofetilide and amlodipine is drug of future. In order to study the possible pharmacokinetic interaction between dofetilide and amlodipine in study animals a unique sensitive HPLC assay that is able to quantify both drugs was then required. Several analytical methods for quantifying dofetilide by HPLC [16] and RIA [17] or amlodipine by HPLC [18-22], HPLC with radioactivity detection [23]and LCMS [24],in biological fluids have been reported. A method is reported for simultaneous determination of dofetilide and amlodipine in plasma by using fluorescence detector [15] but it uses gradient program for elution having longer run time along with tedious sample preparation procedure. In contrast, this article describes a rapid and sensitive HPLC-FL detection method for simultaneous determination of blood concentration of dofetilide and amlodipine, on dried blood spots. FL detector gives better sensitivity for analysis of analyte in biological fluids.[25-28].

Dried blood spots (DBS), the collection of blood samples on a filter paper, is a useful technique for screening in–born errors of metabolism. It is superior to venous sampling because no skilled person is required for sample collection and requires very less blood volume, therefore gaining significance in therapeutic drug monitoring and pharmacokinetics.[29-30].

In the present work we are describing the development and validation of bio-analytical method for simultaneous determination of dofetilide and amlodipine from dried blood spot by using liquid chromatography with fluorescence detector, which provide efficient separation and sensitivity. Design of experiment (DOE) was applied to study the robustness of method. The method was validated according to the ICH guidelines.[31-32].

MATERIAL AND METHODS

Chemicals and Reagents

DFT and AML were obtained as gratis sample from Aurobindo Pharma ltd Hyderabad, India. HPLC grade water from Milli-Q (Millipore, Merck, USA), HPLC-grade acetonitrile, ammonium acetate, ammonium formate and acetic acid from (Rankem India Pvt Ltd.). Polypropylene tubes (Tarsons , India) were used. The repeater multi pipette used for spotting blood were obtained from Eppendorf Research (Sigma

Aldrich, India). The Harris punch and cutting mat were supplied by Whatman (Sanford, USA). FTA blood spot cards were supplied by Whatmann (Sanford, ME, USA).A micro-centrifuge (force 1418) supplied by Select Bio-Products (Edison, NJ) and vortex (Maxi Mix II) from Thermo Scientific India were used. EDTA-coated capillaries (Sarstedt, Leicester, UK) were used. Sachets of silica gel and sealing plastic bags for the storage of blood spot cards were purchased from the local market.

Instrumentation

HPLC-FL analysis was performed using a Agilent Technology (Model 1260 Infinity series) equipped with degasser, quaternary pump (G1311C), autosampler (G1329B), column oven compartment (G1316A), fluorescence detector (G1321B) and diode array detector (G1315D), The pH measurements were carried using Labindia, Model PICO^{+},. The chromatographic and the integrated data were recorded with HP-Vectra computer system as workstation using Chemstation as data acquiring software. All analyses were performed in triplicate and the average response considered.

Chromatographic Conditions

Chromatographic separation was achieved on AkzoNobel, Kromasil 100 A^{o}C8 (250 mm × 4.6 mm, 5 µm) column under isocratic mode of elution. The mobile phase was a mixture of 20mM ammonium acetate (pH 5.0) and acetonitrile (60:40, v/v) pH adjusted to 5.0 with acetic acid. The mobile phase was freshly prepared, filtered through a Millipore filter (pore size 0.45 mm) and degassed continuously using an on-line degasser. Separation was performed at 40 ^{o}C column oven temperature using a 1.0mL min^{-1} flow rate and 10.0 min run time. The injection volume was 20 µL and the detection was performed using Fluorescence detector for DFT 240 nm excitation/370 nm as emission and for AML 240 nm excitation/460 nm as emission with gain of 18 PMT (Photomultiplier Tube).The retention time obtain for DFT and AML were 3.22 and 8.48 min respectively and found reproducible throughout all experiments.

Preparation of Working Standard Solutions

Primary stock solution of DFT and AML were prepared by dissolving accurately 25 mg of DFT and 50 mg of AML in 50mL of diluent (Acetonitrile: water in the ratio of 70:30 v/v) to produce DFT and AML concentration of 0.5 mg mL^{-1} and 1.0 mg mL^{-1} respectively. Aliquot was subsequently diluted to yield a working stock solution of 5000 ng mL^{-1} and 10000 ng mL^{-1}. All the solutions were stored in dark at 5^{0}C and brought to room temperature before use.

Preparation of Calibration Standards (CS) and Quality Control (QC) Samples

The determination of DFT and AML were based on the external standard quantification method. calibration standards were prepared on the day of analysis in the range of 2 to 500 ng mL^{-1} for DFT at concentrations of 2, 5, 10, 20, 40, 100, 200 and 500 ng mL^{-1} and 5 to 1000 ng mL^{-1} for AML at concentrations of 5, 10, 20, 40, 80, 200,400 and 1000 ng mL^{-1} by Spiking appropriate aliquots of working solutions to blank pooled drug-free rat blood EDTA coated capillaries were used for collection of bood. The QC samples at concentrations of 3 (LLQC) ,15 (Low Quality control, LQC)), 150 (Middle Quality control, MQC)), and 300 (High Quality Control, HQC)) ng mL^{-1} and 6 (LLQC), 30 (Low Quality control, LQC)), 300 (Middle Quality control, MQC)), and 600 (High Quality Control, HQC)) ng mL^{-1} for DFT and AML were prepared respectively. Spiked blood was vortex for 5 min and kept for 15 min at room temperature before spotting onto the FTA cards to allow the even distribution of sample.

Blood Spotting

The DBS spot were prepared by spotting 30 µL of respective spiked calibration standards, quality control samples or whole blood obtained from the rats onto pre-drawn 15 mm circles of FTA cards with a calibrated micro- pipette and allowed to dry in dark for 3 h prior to analysis, or stored at 5^{o}C in a sealed plastic bag containing desiccant until analysis.

DBS Processing and Extraction Procedure

The samples were extracted from FTA card by punching 8.0 mm i.d. disk from the centre of the DBS into a clean polypropylene tube. It was then extracted by the addition of 1000 µL Acetonitrile: water (80:20) containing 0.1 % acetic acid (extraction solution) followed by vortex mixing for 5 min and sonication for 3 min. After sonication, the tubes were centrifuged for 15 min at 5000 g, after processing the supernatant was transferred to a clean HPLC vial for analysis.

Application of Method

All animal experiments were undertaken with the approval of 'Institutional Animal Ethical Committee' of Y B Chavan College of Pharmacy; Aurangabad India. Wistar rats (200–220 g) were used for study obtained from Wockhardt Research Centre, Aurangabad India. Throughout the experimental period, the animals were housed under standard conditions in cages at 20 ± 2 ^{0}C with relative humidity 50 ± 15% at animal house facility of Y B Chavan College of Pharmacy, Aurangabad, India and were exposed to 12/12 h light–dark cycle. They were fed with standard laboratory diet supplied by M/S. Krishna valley Agrotech LLP, Pune, India. Food and water was allowed *ad libitum* during the experiment. Six Wister rat with

similar weight and age were utilized to carry out the present pharmacokinetics study After a single dose by oral administration of 2 mg kg^{-1} of DFT and 5 mg kg^{-1} AML to healthy Wistar rats (n = 6), blood samples were collected for the determination of DFT and AML concentrations. into the processed test tube coated with EDTA at initial and after every 30 min for up-to 12 h of post-dose and spotted on to cards with drug samples. Dried blood spots (DBS) were extracted and the concentrations of DFT and AML were determined against the calibration curve on the day of analysis. 'Phoenix Winnonlin', version 7 software was used for calculation of pharmacokinetics parameters.

RESULTS AND DISCUSSION

Method Development

In view of pharmacokinetic investigation the purpose of this work was to develop the sensitive and reliable bio-analytical method for simultaneous quantification of DFT and AML on dried blood spot. This study would be beneficial for understanding the interaction between these drugs and therapeutic monitoring of drug levels. The reported method has few disadvantages like long run time with gradient mode of elution. Therefore the goal was to developed a short method, so that more number of samples can be analyzed per day. Initially, different analytical columns like Inertsil ODS 3V, YMC-Pack ODS AM, Zorbax Eclipse XDB C8 and AkzoNobel, Kromasil 100 A°C8 were tried and their chromatographic performance was compared for their peak shape, retention time, column efficiency and respective responses. Even with different compositions of mobile phase the peak shape for DFT was not symmetrical, addition of acetic acid played an important role in improving the peak shape of DFT. Among all tested columns AkzoNobel, Kromasil 100 A°C8 (250 mm × 4.6 mm, 5 µm) column was chosen due to its highest signal response, symmetric peak shape, higher number of theoretical plates and acceptable retention times for quantification of DFT and AML. The mobile phase composition was optimized with mixtures of different pH buffers and acetonitrile/methanol at different ratios. Finally ammonium acetate pH 5 ± 0.5 with acetic acid was selected. The final opted mobile phase was a mixture of buffer: acetonitrile in proportion of 60:40 v/v which provided good peak shape, separation and optimum retention time. For optimizing the fluorescence detection parameters, data acquisition mode was kept as fluorescence mode. Ex and Em wavelength were optimized by scanning the excitation spectra from 200 to 350 nm and emission spectra from 300 to 800 nm with wavelength step of 5 nm. For DFT the Ex. and Em. wavelengths were found to be 240 and 370 nm. For AML the Ex. and Em. wavelengths were found to be 240 and 460 nm. The PMT gain was kept at 18.

Method Validation

The developed method was validated according to ICH and FDA guidelines [31-32]

Specificity and Selectivity

The specificity and selectivity of the method were evaluated by comparing chromatograms of drug free rat dried blood (n=6) and spiked dried blood processed samples by determining the interference of endogenous co-eluent components at the retention time of both the analytes. Figure. 2a shows the typical chromatogram obtained under the above-mentioned conditions. The spiked chromatograms at LLQC, LQC and HQC level were shown in Figure 2b,2cand 2d. The chromatogram of a blood sample obtained from the rats under pharmacokinetics study after oral dose of administration was shown in Figure 3b and Chromatogram of blood sample obtain from rat under study was shown in Figure 3a.

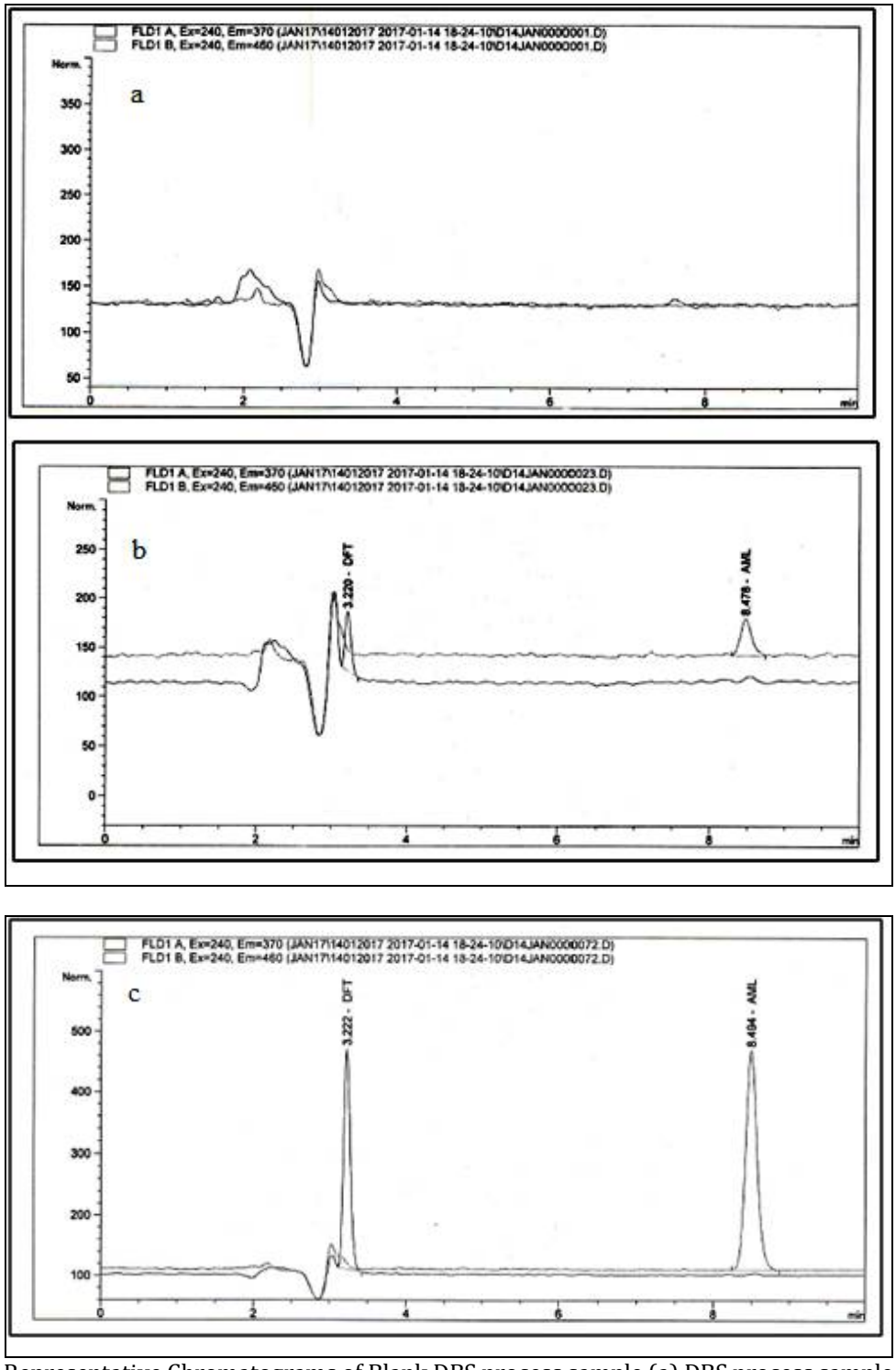

Figure.2 Representative Chromatograms of Blank DBS process sample (a) DBS process sample spiked at LQC level (b) and DBS process sample spiked at HQC level (c)

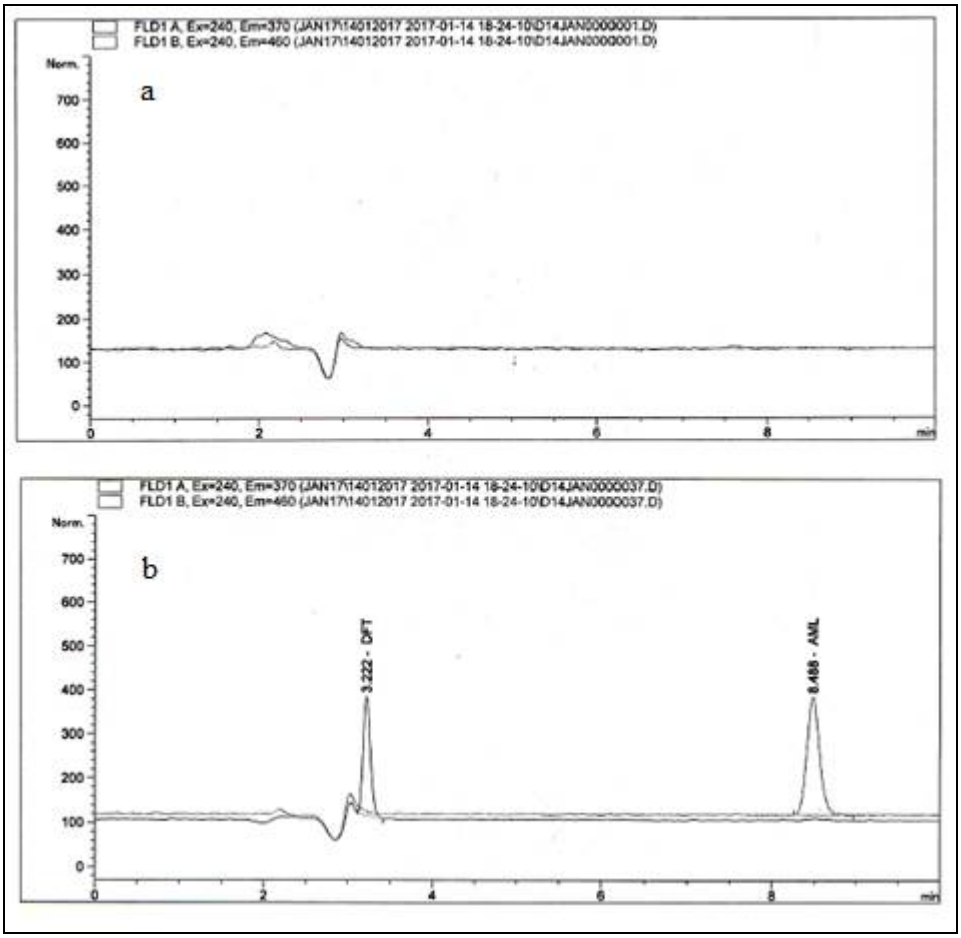

Figure.3 Representative Chromatograms of blood obtain from wister rats after oral administration of DFT and AML DBS process sample after initial time point (a) DBS process sample after C_{max} level (b)

Linearity

The linearity of the method was evaluated by running DBS standard over the range of 2 to 500 ng mL^{-1} for DFT and 5 to 1000 ng mL^{-1} for AML respectively, the calibration curve was generated by plotting peak area against drug concentrations. Regression analysis showed the correlation coefficient of r= 0.999 for both analytes with regression equation of y=6.622x-34.23 for DFT and y=2.383x-10.80 for AML, which indicates the excellent linearity over the experimental concentration range as shown in Table-1

Table 1 Summary of Linearity Data

Parameter	DFT	AML
Linearity Range (ng mL^{-1})	2-500	5-1000
Equation Parameter		
Slope	6.622	2.383
Intercept	-34.23	-10.80
Standard Deviation (Slope)	0.01	0.03
Standard Deviation (Intercept)	0.87	0.76
%RSD (Slope)	0.08	0.88
%RSD (Intercept)	-1.12	-1.56
correlation coefficient (r)	0.999	0.999

Limit of Detection and Quantification

The limit of detection and quantification (LOD and LOQ) were calculated using calibration curve method by measuring the standard deviation of the response (σ) and the slope (S) of the calibration curve, by applying the equation as limit of detection LOD = 3.3 (σ/S) and limit of quantitation (LOQ) = 10 (σ/S). The LOD and LOQ of DFT and AML were 0.7, 1.6 ng mL^{-1} and 1.2, 3.8 ng mL^{-1} respectively, indicating high sensitivity of the developed method.

Precision and Accuracy

The intra-day, inter-day precision and accuracy were investigated by measuring the quality control samples at four different levels (3,15,150 and 300 ng mL^{-1} for DFT and 6,30, 60 and 600 ng mL^{-1} for AML) in six replicates. Precision was evaluated by determining %CV and accuracy from recovery or bias from true value of analyte. All results obtained were within acceptance limit as summarized in Table 2. The assay precision was ranged from 0.79 to 7.41 % for DFT and 0.75 to 5.08 % for AML for intra and inter day including all experimental levels and accuracy was in the range of 90.41 to 100.10 % at all levels for both analytes.

Table 2 Intra-day and inter-day precision and accuracy of DFT and AML of DBS samples (n=6)

QC samples	DFT				AML			
	LLQC	LQC	MQC	HQC	LLQC	LQC	MQC	HQC
Theoretical Concentration (ng mL^{-1})	3	15	150	300	6	30	300	600
	Intra-day Precision and accuracy							
Mean observed concentration (ng mL^{-1})	2.87	13.84	145.82	300.31	5.80	28.83	295.06	591.19
SD	0.25	1.03	3.52	3.34	0.31	1.23	6.94	4.86
Precision (%CV)	8.64	7.41	2.41	1.11	5.34	4.26	2.35	0.82
Accuracy (%)	95.78	92.24	97.22	100.10	96.72	96.09	98.35	98.53
Accuracy (Bias)	-0.13	-1.16	-4.18	0.31	-0.20	-1.17	-4.94	-8.81
	Inter-day Precision and accuracy							
Mean observed concentration (ng mL^{-1})	2.85	13.56	147.06	299.58	5.93	29.06	295.25	594.03
SD	0.20	0.61	3.68	2.36	0.28	1.48	6.59	4.43
Precision (%CV)	7.10	4.53	2.50	0.79	4.67	5.08	2.23	0.75
Accuracy (%)	95.10	90.41	98.04	99.86	98.75	96.88	98.42	99.00
Accuracy (Bias)	-0.15	-1.44	-2.94	-0.42	-0.07	-0.94	-4.75	-5.97

Recovery and Matrix effect

The extraction recovery for DFT and AML in DBS were evaluated by comparing the peak area of an analytes of a DBS process sample (n=6) with those obtained by calibration standard solution of the same concentration . To determine recovery, 30 µL spots were made and allowed to dry. The overall recovery obtain at four QC levels were found to be greater than 85% as shown in Table-4. The matrix effect was assessed (n = 6) by analyzing LLQC, LQC, MQC and HQC samples. To determine the matrix effect, initially blank DBS process sample were analyzed; aqueous solutions of analytes of the same concentrations were prepared and analyzed. The matrix factor was 0.92-0.99 (LQC) and 0.91-1.00 (HQC) for DFT and 0.89-0.98 (LQC) and 0.90-1.05 (HQC) for AML. It indicates that there is no endogenous interference at retention time of analytes.

Dilution Integrity and Carry-Over Effect

To demonstrate that a method is suitable for a DBS sample with analyte concentration higher than upper limit of the calibration range. Blood samples containing DFT and AML at the concentrations of 2000 and 3000 ng mL^{-1}, respectively, were prepared. These samples were diluted by 5-fold and 6-fold of the original concentration using whole blood. The dilution integrity was evaluated from accuracy which was 90-105 % with CV of ≤10.0%. Carry-over effect, was evaluated by injecting two extracted blank DBS samples sequentially after a higher limit of quantification (HQC) sample injection, No carryover effect was observed.

Stability

The stability of analytes at various conditions were evaluated by analyzing DBS spiked samples exposed to different conditions (time and temperature) at four QC concentration levels (3,15, 150 and 300 ng mL^{-1} for DFT and 6,30, 60 and 600 ng mL^{-1} for AML) in six replicates. These results were compared with those obtained from freshly prepared DBS samples. Freeze–thaw stability was evaluated after three complete freeze–thaw cycles (-20 to 25°C) on consecutive days. Short-term temperature stability was assessed by analyzing samples that were kept at ambient temperature (25 °C) for 6 hrs. Auto-sampler stability was evaluated by analysis of processed and reconstituted quality control samples, which were stored in the autosampler tray for 12 hrs at 10°C. Long-term stability was performed by analyzing samples that were stored in at frozen state in (-20°C) for 30 days. For each concentration and storage condition, six replicates were analyzed in one analytical batch. The concentration after each storage period was calculated against calibration curve obtained from freshly prepared standards. Table 3 shows short- and long-term stability, three freeze–thaw cycles and 6 h room temperature storage for quality control samples, indicating that DFT and AML was stable in the dried blood sample under the experimental conditions. The results were found to be well within the acceptance limits.

Robustness by using Design of Experiment (DoE)

The robustness of the HPLC method was evaluated by DoE technique, according to the ICH guideline Q2A. An augmented Plackett–Burman, experimental design was applied, to study the influence of the internal parameters such as percentage of acetonitrile, buffer concentration, flow rate, column oven temperature and pH. A Plackett–Burman design is an orthogonal two-level experimental design that can be used to fit linear models. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, deliberate variations in method parameters and provides an indication of its reliability during normal usage. Three different types of method parameters exist: basic, internal and external parameters. The robustness study was limited to investigating the influence of basic and internal parameters. The experiments were run randomly with a DBS sample spiked with 100 ng mL^{-1} of DFT and 200 ng mL^{-1} of AML. The resolution (*Rs*), retention time (t_R), Theoretical plate count and tailing factor (T_f) were evaluated as responses. Plotting the coefficient (scaled and centered) plots for all the observed responses revealed that logarithmic transformations were necessary for optimizing the responses. The respective 95% confidence intervals are shown as error bars. Coefficient with 95% confidence interval including zero were statistically insignificant. The residual plot of observed versus predicted responses revealed the linear relationship between parameter studied. It was observed that different combinations of significant parameters do not drastically affect responses, based on this developed method was considered to be robust.

Table 3 Stability data of DFT and AML on dried blood spot (n=6)

DBS samples		DFT				AML			
		Concentration in ng mL^{-1}							
		3	15	150	300	6	30	300	600
Initial	Mean	2.85	14.11	146.43	293.37	5.93	29.68	297.62	592.15
	SD	0.20	0.67	2.48	5.60	0.28	0.58	1.23	3.96
	%CV	7.10	4.75	1.69	1.91	4.67	2.03	0.41	0.67
	Accuracy (%)	95.10	94.07	97.62	97.79	98.75	96.21	99.29	98.69
6 hours at 25^0C (Short term stability)	Mean	2.89	13.98	145.12	294.12	5.95	28.96	296.98	590.69
	STDV	1.02	1.02	0.96	6.60	0.69	0.69	1.98	4.98
	%CV	6.98	6.98	2.89	2.11	2.25	2.25	0.98	1.02
	Accuracy (%)	96.33	93.20	96.75	98.04	99.17	96.53	98.99	98.45
	%Drug Remaining	101.30	99.08	99.11	100.26	100.42	97.57	99.78	99.75
30 days at -20^0C (Long term stability)	Mean	2.91	14.25	146.87	294.98	5.98	29.98	298.85	596.69
	STDV	1.02	1.02	0.96	6.60	0.69	0.98	2.02	5.23
	%CV	6.98	6.98	2.89	2.11	2.25	1.98	0.65	0.98
	Accuracy (%)	2.91	95.00	97.91	98.33	5.98	99.93	99.62	99.45
	%Drug Remaining	100.69	101.93	101.21	100.29	100.50	103.52	100.63	101.02
Four freeze thaw cycles (Freeze-thaw stability)	Mean	2.87	13.69	144.99	293.98	6.13	28.92	297.36	593.89
	STDV	1.02	1.02	0.96	6.60	0.69	0.98	2.02	5.23
	%CV	6.98	6.98	2.89	2.11	2.25	1.98	0.65	0.98
	Accuracy (%)	2.83	91.27	96.66	97.99	6.10	96.40	99.12	98.98
	%Drug Remaining	98.63	96.07	98.72	99.66	102.51	97.44	99.91	100.29
SD :Standard deviation	%CV : Precision	* Average of Six Determination (n=6)							

Impact of Blood volume spotted

To study the effect of blood volume spotted on FTA card , we plotted 25, 30 and 35 µL DBS samples at concentration of 15,300 ng mL^{-1} for DFT and 30,600 ng mL^{-1} for AML were prepared in replicate (n = 6). A 8 mm diameter disc was punched from the centre of each sample and extracted. The concentrations of extracts were determined by using the linear regression equation generated from a calibration produced from 30 µL DBS samples. The precision was within the 15% limit for 25 and 35 µL spot sizes at the two tested concentrations. Indicating that the amount of blood spotted did not significantly affect the distribution of DFT and AML across the FTA card for drug quantification

Application to Pharmacokinetics in Rats

The developed HPLC-FL method was successfully used to investigate the pharmacokinetic parameters in Wister rat (n=6) after oral administration of 2.0 mg kg^{-1} DFT and 5.0 mg kg-1 AML. Blood concentrations were determined up to 12 h post dose. At every 30 min time interval about 100 µL of blood was withdrawn from the epicanthic veins and transferred to EDTA-coated tubes. Exact volume of 30 µL of blood was spiked on the DBS card and extracted as per procedure. The extracted samples from DBS were analyzed against calibration standards and QC samples to monitor the system performance as one batch in a single analytical run. The standard calibration curve, including blank sample and standard zero samples, was used to determine the sample concentrations in the unknown DBS samples. QC samples (at each of low, medium and high concentrations) were analyzed together with the unknown DBS samples.

The pharmacokinetic parameters were calculated using 'Phoenix Winnonlin' version 7 software and were summarized in Table 5. A pharmacokinetic plot obtained under studied condition was shown in Figure. 4. Thus, the present developed HPLC assay method could be successfully applied to the determination of DFT and AML in several preclinical and clinical studies conducted in any institution.

Table 4 Recovery of DFT and AML

DFT								
	LLQC (3 ng mL^{-1})		**LQC (15 ng mL^{-1})**		**MQC (150 ng mL^{-1})**		**HQC (300 ng mL^{-1})**	
	Found	% Recovery	Found	% Recovery	Found	% Recovery	Found	% Recovery
Mean	2.87	95.78	13.56	90.41	147.06	98.04	299.58	99.86
STDV	0.25	8.27	0.61	4.09	3.68	2.46	2.36	0.79
Precision (% CV)	8.64	8.64	4.53	4.53	2.50	2.50	0.79	0.79
AML								
	LLQC (6 ng mL^{-1})		**LQC (30 ng mL^{-1})**		**MQC (300 ng mL^{-1})**		**HQC (600 ng mL^{-1})**	
	Found	% Recovery	Found	% Recovery	Found	% Recovery	Found	% Recovery
Mean	5.80	96.72	29.06	96.88	295.25	98.42	594.03	99.00
STDV	0.31	5.16	1.48	4.93	6.59	2.20	4.43	0.74
Precision (% CV)	5.34	5.34	5.08	5.08	2.23	2.23	0.75	0.75
* Average of Six Determination (n=6)								

Table 5 Pharmacokinetic parameters of DFT and AML in rats (n=6) after oral administration

Parameter	**DFT**	**AML**
Maximum concentration C_{max} (ng mL^{-1})	20.8	58.0
Time required to reach maximum concentration Tmax (h)	4.0	2.5
Area under blood concentration-time curve $AUC_{0\ to\ t}$ (ngmL^{-1}h^{-1})	96.55	285.28
Area under blood concentration-time curve $AUC_{0\ to\ \infty}$ (ngmL^{-1}h^{-1})	105.29	312.69
Half life $t_{1/2}$ (h)	2.86	3.04
Elimination rate constant K_{el} (1/h)	0.24	0.23
$AUC_{0\ to\ t}$ / $AUC_{0\ to\ \infty}$	0.92	0.91
Mean residence time MRT (h)	5.25	4.96
Clearance, Cl (mLh^{-1}kg^{-1})	47688.8	15853.3

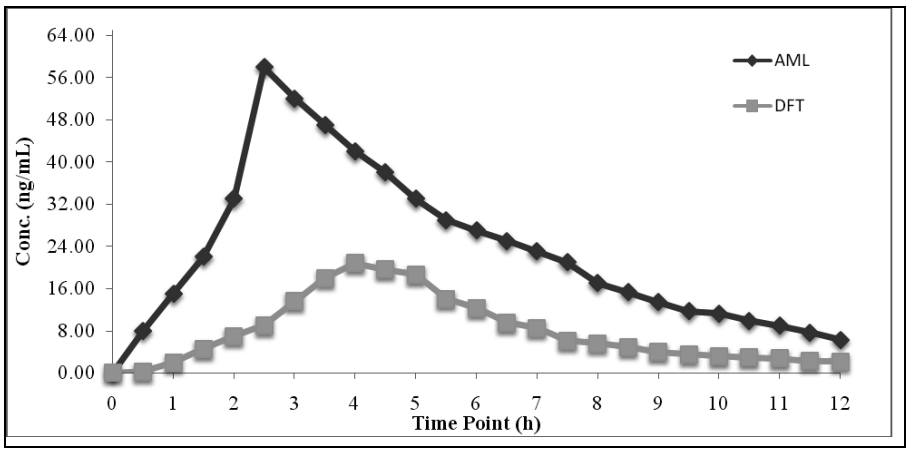

Figure .4 Pharmacokinetic plots obtained after oral administration of 2 mg kg^{-1} of DFT and 5.0 mg kg^{-1}ofAML to rats under study

CONCLUSION

A novel simple, reliable and fast LC-FLD method was developed and validated to determine the concentration of DFT and AML on dried blood spot. Compared to previously reported method this method has short run time with simple sample processing which allows high sample throughput. It was successfully applied to monitor pharmacokinetic parameters in rats after single oral dose administration. It has sufficient sensitivity with limit of quantification of 1.6 and 3.8 ng mL^{-1} for DFT and AML respectively, subsequently it can be extended to study the concentration of both drugs in human to understand pharmacokinetic profile and during bioequivalence studies. The method described here is suitable for clinical monitoring and management of clinical and pharmacokinetics research, even in institutions that are particularly not well equipped

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

ETHICAL APPROVAL
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Having Approval number CPCSEA/IAEC/Pharma.Chem-30/2016-17/127

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