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Hydrophilic interaction liquid chromatography for the determination of vesnarinone on dried blood spots; application to pharmacokinetics in rats

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A highly selective, sensitive and rapid hydrophilic interaction liquid chromatographic method was developed and validated for determination of vesnarinone on dried blood spots. Naftopidil was used as an internal standard. The chromatographic separation was achieved on a reversed-phase zwitterionic hydrophilic interaction liquid chromatographic ZICWHILIC-C18 (4.6 × 100 mm; 5 mm) column using acetonitrile: 10 mM potassium dihydrogen phosphate (pH 4.0; 70 : 30, v/v) as a mobile phase in an isocratic elution mode at a flow rate 0.8 mL min^{-1} at 27 °C. Photodiode array detection wavelength was set at 247 nm to monitor the column eluents. The method was validated for accuracy, precision, linearity and selectivity by design of experiments following ICH guidelines. The assay exhibited a linear range of 30–2000 ng mL^{-1} for vesnarinone on dried blood spots. The lower limit of detection was found to be 30 ng mL^{-1}. The intra- and inter-assay coefficients of variation did not exceed 11.46% deviation of the nominal concentration. The recovery of vesnarinone from dried blood spots was >95.0% and its stability were excellent with no evidence of degradation during sample processing for at least 3 months storage in a freezer at −20 °C. The method was successfully applied to a pharmacokinetic study of vesnarinone in rats.

1 Introduction

Vesnarinone (VSN) (6-[4-(3,4-dimethoxybenzoyl)piperazin-1-yl]-3,4-dihydro-1*H*-quinolin-2-one) is a cardio-tonic agent (Fig. 1(a)). VSN, a quinolinone derivative, is an oral inotropic agent that augments myocardial contractility in modal systems, with little effect on the heart rate or myocardial oxygen consumption.[1] It is a mixed phosphodiesterase three inhibitor and ion-channel modifier that has modest dose-dependent, positive inotropic activity, but minimal negative chronotropic activity.[2] Vesnarinone was shown in a short-term placebo-controlled trial to improve survival markedly in patients with severe heart failure when given at a dose of 60 mg per day, but there was a trend toward an adverse effect on survival when the dose was 120 mg per day. Naftopidil (NTP) was used as an internal standard (IS) for the quantification of VSN (Fig. 1(b)). HPLC and LC-MS/MS method were developed for measuring vesnarinone and its metabolites quantitatively.[3,4] Vesnarinone can form several metabolites either by the cleavage of the piperazine moiety at the amide bond or by hydroxylation of the piperazinyl ring.[5] Subsequently, a more specific and sensitive method was developed for measuring vesnarinone and its metabolites.[6] To the best of authors' knowledge, methods for determination of VSN on rat dried blood spots have not been reported in the literature and until now no RP-HPLC method has been reported for the validation of VSN.

Zwitterionic hydrophilic interaction liquid chromatography (ZICWHILIC-C18) has been widely recognized as a distinct chromatographic mode and has enjoyed nearly a decade of rapid growth since its potential in separating very polar compounds was rediscovered in the early 2000s.[7,8] It is a technique for the separation of polar and hydrophilic compounds using polar stationary phases in conjunction with mobile phases consisting of a polar organic solvents (typically acetonitrile) containing an appreciable concentration of water. Reasonable peak shapes, enhanced detector sensitivity and direct injection of extracts owing to the high organic content in the mobile phase are some of the advantages of this technique.[9–11]

Dried blood spots (DBS) introduced by Guthrie *et al.* are an innovative blood sampling technique involving the collection of small volumes of human blood from heel/finger pricks, spotting on an appropriate filter paper, drying, and transporting to the laboratory for analysis.[12] Initially it was used for neonatal screening of phenylketonuria. Now it is successfully adopted and widely practised by clinical labs in newborn screening for inherited metabolic disorders. It requires a micro-volume

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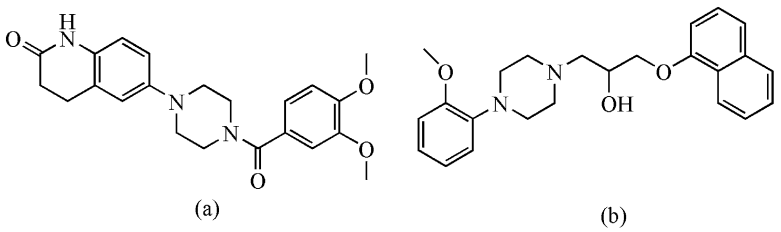

(a) (b)

Fig. 1 Chemical structures of (a) vesnarinone (VSN) and (b) naftopidil (NTP, IS).

(typically $\leq$ 50 μL) blood sample for drug quantification. Recently, it has gained interest in drug discovery and development processes due to the fact that it offers several advantages over conventional whole blood, plasma or serum sample collection.[13–16]

The present manuscript describes the development and validation of a simple, rapid, sensitive and selective HILIC method for determination of VSN on dried blood spots. Its application to pharmacokinetics in rats is also demonstrated.

2 Experimental

2.1 Chemicals and reagents

VSN and NTP were obtained from a local manufacturing unit in Hyderabad. Glass-distilled de-ionized water (Nanopure, Barnstead, USA), HPLC-grade methanol and acetonitrile (SD Fine Chemicals, Mumbai, India), and AR-grade orthophosphoric acid (Sisco Research Laboratories Pvt. Ltd, Mumbai, India) were used. Sample tubes and the repeater multipipette used for spotting blood were obtained from Tarsons (Kolkata, India). The Harris punch and cutting mat were supplied by Whatman (Sanford, USA). A centrifuge (model 2-16P) supplied by Sigma (Zurich, Switzerland) was 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.

2.2 Instrumentation

The HPLC system consisting of a quaternary LC-20AD pump, a SPD-M20A diode array detector, a SIL-20AC auto sampler, a DGU-20A5 degasser and CBM-20A communications bus module (all from Shimadzu, Kyoto, Japan) was used. The pH measurements were carried out by Elico, model LI 120, pH meter equipped with a combined glass–calomel electrode. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan). Separation and quantitation were made on a ZICWHILIC-C18 (4.6 × 100 mm; 5 mm) (Merck, Darmstadt, Germany).

2.3 Animals

Wistar rats (200–220 g) were procured from M/S. Mahavir enterprises, Hyderabad, India. The use of animals was approved by the 'Institutional Animal Ethical Committee' (169/99/CPCSEA, University College of Pharmaceutical Sciences, Warangal, India). All the experiments were done based on laws and guidelines. Throughout the experimental period, the animals were housed under standard conditions in cages at room temperature (20 ± 2 °C), relative humidity (60–70%) and were exposed to 12/12 h light–dark cycle. They were fed with standard laboratory diet supplied by M/S. Rayans Biotechnologies Pvt. Ltd, Hyderabad, India. Food and water was allowed *ad libitum* during the experiment. After a single dose by oral administration of 30 mg kg^{-1} of VSN to healthy Wistar rats (n = 6), blood samples (1 mL) were collected for the determination of VSN concentrations. Serial blood samples were collected into the processed test tube at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 24 h post-dose and spotted on FTA cards with drug samples. Dried blood spots (DBS) were extraction sonicated followed by centrifugation at 4500*g* for 20 min and stored frozen at −20 °C. Specimens were thawed and allowed to reach room temperature, and the concentrations of VSN were determined from the calibration curve on the same day. Statistical analysis was performed using Microsoft Excel 2003 while 'Ramkin', software (Drug and Statistics, Mathematical Pharmacology Professional Committee of China, Shanghai, China) was used for calculation of pharmacokinetic parameters.

2.4 Preparation of stock solutions, calibration standards (CS) and quality control (QC) samples

Stock standard solutions of VSN and the IS were prepared in methanol at a concentration of 1 mg mL^{-1} and stored at 5 °C. Working standards of VSN were prepared by appropriate dilutions of stock solution with methanol. Similarly NTP (IS) working standard was also prepared by diluting the stock solution in methanol and used as required. The DBS calibration standards were prepared at concentrations of 30, 60, 120, 200, 500, 1000, 1500 and 2000 ng mL^{-1} of VSN and 30 ng mL^{-1} of IS by spiking appropriate aliquots of working solutions to blank pooled drug-free rat blood. The quality control samples at concentrations 90 (Low Limit of Quality Control (LLQC)), 300 (Low Quality control (LQC)), and 1600 (High Quality Control (HQC)) ng mL^{-1} were prepared in a similar fashion. Spiked blood was kept for 30 min at room temperature before spotting onto the FTA cards to allow the even distribution of VSN in the sample. Appropriate volumes of VSN working standard solution (100 μg mL^{-1}) were added to drug-free dried blood spots (30 mm) to prepare eight non-zero standard drug concentrations (30, 60, 120, 200, 500, 1000, 1500 and 2000 ng mL^{-1}), and three quality control concentrations (90, 300 and 1600 ng mL^{-1}). Standard drug concentrations used for preparation of the calibration curves were different from those employed in the quality control studies.

2.5 Dried blood spots (DBS) and extraction procedure

The DBS were prepared by spotting 30 μL of the respective spiked CS/QC or whole blood from the VSN treated rats onto

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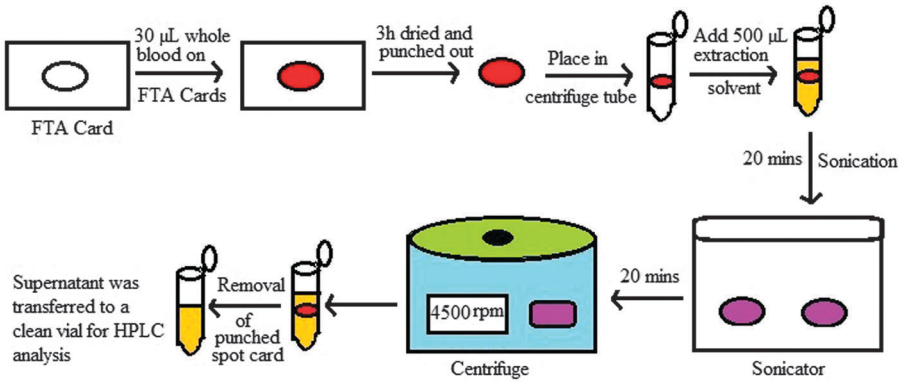

Fig. 2 Dried blood spot (DBS) sample extraction procedure.

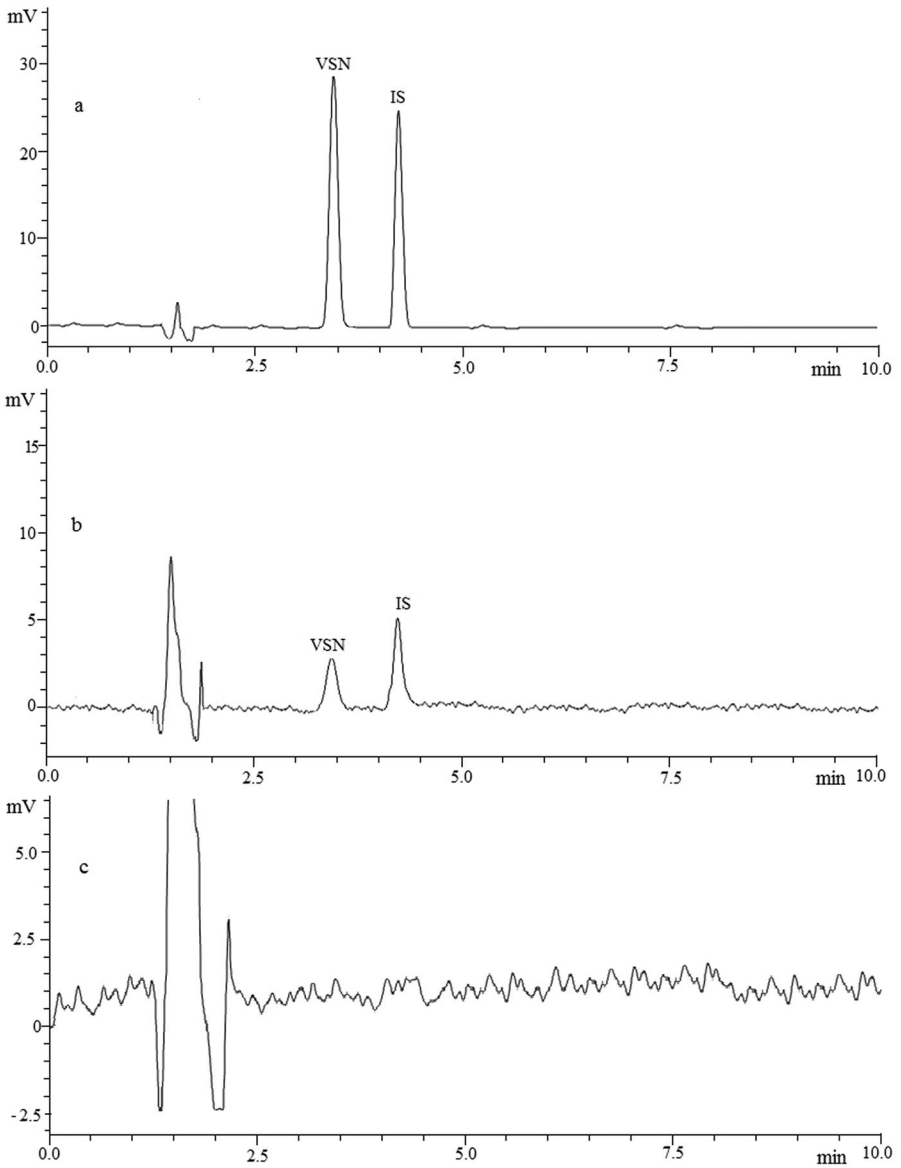

Fig. 3 Representative *in vitro* chromatograms of DBS samples spiked with VSN and IS at (a) higher limit of quantitation, (b) lower limit of quantitation and (c) blank.

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sampling paper using a calibrated pipette. The samples were left to dry in the dark for at least 3 h before storing in a sealed plastic bag containing desiccant until analysis. The method of extraction is shown in Fig. 2. A 3.0 mm disk was punched from the centre of the DBS into a clean tube. It was then extracted by the addition of 500 μL extraction solution (methanol) followed by vortex mixing for 5 min and sonication for 20 min. After sonication and centrifuging for 20 min at 4500 *g*, the supernatant was transferred to a clean tube for HPLC analysis.

3 Results and discussion

3.1 Method development

Initially, different compositions of mobile phases with different columns were tried, but the peaks were not separated with good resolution. Longer retention times, weak separations and poor resolutions were observed by using different mobile phase conditions with Lichrocart and Xterra C18 columns. Finally, chromatographic separation was achieved on ZICWHILIC-C18 (4.6 × 100 mm; 5 mm) (Merck, Darmstadt, Germany). The mobile phase was a mixture of 10 mM potassium dihydrogen phosphate at (pH 4.0) and acetonitrile (30 : 70, v/v), pH was adjusted to 4.0 with orthophosphoric 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 room temperature using a 0.8 mL min^{-1} flow rate and 10 min run time. The injection volume was 5 μL and the detection wavelength was set at 247 nm. Fig. 3 shows the representative chromatograms of blank dried blood samples spiked with VSN and IS at (a) higher limit of quantitation, (b) lower limit of quantitation and (c) blank. Fig. 4 shows the chromatograms of DBS samples obtained from Wistar rats following an oral dose of 30 mg of VSN with IS (a) after 1.90 h and (b) at zero time. The analytes were well separated from DBS samples. The retention times of VSN and IS were 3.517 and 4.362 minutes, respectively (Fig. 4). The total run time was 10 min. The collected sample was sufficient to isolate the VSN and IS from DBS without any interfering endogenous peaks at 247 nm.

3.2 Method validation

The described method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, specificity, stability, precision and accuracy according to FDA and ICH guidelines.[17,18] The results are reported in Table 1.

3.2.1 Linearity, selectivity and sensitivity. The peak area ratio of VSN to IS in DBS sample was linear with respect to the analyte concentration over the range 30–2000 ng mL^{-1}. The mean linear regression equation of calibration curve for the analyte was $y = 784.57x + 0.1543$, where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The correlation coefficient (r^2) for VSN was 0.9987 over the concentration range used. A calibrated plot of analyte/IS peak area ratio against nominal VSN concentration was produced and an equally weighted linear regression was applied. The lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The lower limit of detection was found to be 30 ng mL^{-1} for the analyte in

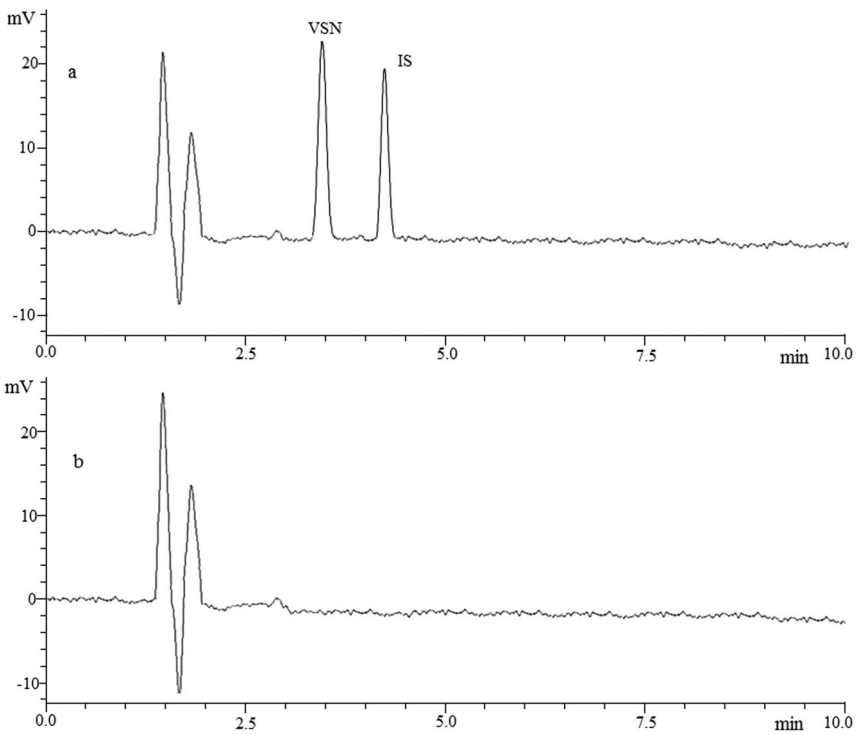

Fig. 4 Representative *in vivo* chromatograms of DBS samples obtained from Wistar rats following an oral dose of 30 mg of VSN with IS (a) after 1.90 h and (b) at zero time.

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Table 1 Back-calculated VSN concentration of the calibration standards on dried blood sample

Nominal concentration (ng mL^{-1})	Mean[a]	Standard deviation (SD)	Accuracy (%)	Precision (%)
30	30.15	3.43	100.50	2.47
60	59.88	8.96	99.80	1.46
120	121.26	15.47	101.05	3.58
200	204.11	18.66	102.05	2.96
500	485.28	25.81	97.06	6.77
1000	981.74	27.56	98.17	5.98
1500	1524.66	42.57	101.64	4.29
2000	2084.23	97.85	104.21	5.63

[a] Average of six determinations.

normal DBS samples. Compared to reported methods it was very low.[4,6] The LOD and LOQ were determined at 3 and 10 times the baseline noise, respectively. The LLOQ was defined by the lowest concentration that gave a signal-to-noise ratio equal to or greater than 10 whilst exhibiting an accuracy of $\leq$15%. Table 1 summarizes the back-calculation of VSN concentrations of the calibration standards in DBS samples. Calibration samples were analyzed from low to high concentration at the beginning of each validation run and the other samples were distributed randomly through the run. The calibration curves were obtained by weighted linear regression (weighting factor $1/x^2$) using the Microsoft Excel 2003 software. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards. The selectivity of the method was determined by analyzing DBS samples (n = 1) collected from six individual rat subjects.

3.2.2 Precision and accuracy. Inter- and intra-day accuracy and precision for the assay were determined from the analysis of replicate (n = 6) calibration standards at six VSN concentrations within the range of 30–2000 ng mL^{-1} on three separate days. The accuracy was expressed as the relative error (RE%) and precision as the coefficient of variation (CV%). Values of RE and CV of $\leq$15% at all concentrations were considered acceptable. The results were reported in Table 2. The precision and accuracy at the LLOQ and at low, medium and high concentrations of VSN in DBS were within the acceptable limits. The intra- and inter-day accuracy and precision of VSN QC samples are shown in Table 2. Within- and between-day relative standard deviations (precision, %CV) were less than 11.46 and 10.58% respectively. Within- and between-day relative errors were less than 6.35 and 9.27%, respectively.

Table 3 Relative recovery of VSN

	QC-1 90 (ng mL^{-1})		QC-2 300 (ng mL^{-1})		QC-3 1600 (ng mL^{-1})	
Sample no.	Found	Recovery[a] (%)	Found	Recovery[a] (%)	Found	Recovery[a] (%)
Mean[a]	89.44	99.37	306.34	102.11	1581.27	98.82
SD	9.86	11.25	16.85	5.91	29.51	4.63
Precision (% RSD)	2.53	2.53	6.54	6.54	7.55	7.55

[a] Average of six determinations.

3.2.3 Specificity. There were no interfering peaks present in six different randomly selected of drug-free dried blood samples used for analysis at the retention times of either analyte or internal standard (Fig. 3 and 4).

3.2.4 Recovery. The extraction recovery was determined by comparing the peak areas of each compound after extraction from DBS with those obtained by direct injection of the same concentration of analyte. To determine recovery, 30 μL spots were made and allowed to dry. The overall recovery was assessed at concentrations of 90, 300 and 1600 ng mL^{-1} in replicate (n = 6). The relative recoveries are reported in Table 3.

3.2.5 Ruggedness and robustness. 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. External parameters, such as different laboratories, analysts and instruments, were not included in the study. The ruggedness of the HPLC method was evaluated by design of experimental method, according to the ICH guideline Q2A.[18] An experimental

Table 4 Selected parameters and their variations used to test robustness of the method by design of experiments

Selected parameters and their variations	−1	0	+1
Percentage of mobile phase (A)	65	70	75
Buffer concentration (mM) (B)	5	10	15
pH (C)	3.5	4	4.5

Dummy factor −1 indicates low-level variance, 0 indicates medium-level variance and +1 indicates high-level variance.

Table 2 Intra- and inter-day accuracy and precision of VSN quality control samples

Sample no.	LLOQ 30 (ng mL^{-1})	QC-1 90 (ng mL^{-1})	QC-2 300 (ng mL^{-1})	QC-3 1600 (ng mL^{-1})
Intra-day				
Mean[a] ± SD	30.23 ± 0.12	89.87 ± 7.45	307.25 ± 22.54	1565.22 ± 54.21
Precision (% RSD)	2.55	3.71	8.83	11.46
Accuracy	100.76	99.85	102.41	97.82
Inter-day				
Mean[a] ± SD	29.94 ± 0.15	91.08 ± 5.47	300.44 ± 17.25	1553.67 ± 32.83
Precision (% RSD)	5.82	4.96	8.62	10.58
Accuracy	99.80	101.20	100.14	97.10

[a] Average of six determinations.

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Table 5 Fractional factorial experimental design and responses developed to test the robustness of the method

Exp. no.	Run order	Percentage of modifier	Buffer concentration	pH	R_s	t_R (VSN)	T_f (VSN)
1	7	−1	−1	−1	3.91	3.71	1.52
2	3	1	−1	−1	2.85	3.48	1.43
3	5	−1	1	−1	2.80	3.67	1.33
4	10	1	1	−1	2.92	4.21	1.18
5	15	−1	−1	1	3.86	5.41	1.55
6	12	1	−1	1	2.85	3.89	1.51
7	8	−1	1	1	3.75	3.76	1.39
8	13	1	1	1	3.95	3.32	1.14
9	9	−1	−1	−1	3.47	4.44	1.56
10	11	1	−1	−1	3.80	5.35	1.42
11	14	−1	1	−1	2.97	4.46	1.30
12	17	1	1	−1	2.99	3.84	1.17
13	4	−1	−1	1	3.45	3.91	1.52
14	16	1	−1	1	3.24	4.63	1.36
15	6	−1	1	1	3.14	3.55	1.27
16	2	1	1	1	2.87	3.67	1.11
17	18	0	0	0	2.96	3.51	1.36
18	1	0	0	0	2.83	3.49	1.31

R_s: resolution, t_R: retention time, and T_f: tailing factor.

design, the augmented Plackett–Burman, was applied to study the influence of the internal parameters (percentage of acetonitrile, buffer concentration and pH) given in Table 4. A Plackett–Burman design is an orthogonal two-level experimental design that can be used to fit linear models. The design matrix with the factor settings is shown in Table 5. The experiments were run randomly with a DBS sample spiked with 100 ng mL^{-1} VSN and IS. The selected responses were calculated (resolution (R_s), retention time (t_R), and tailing factor (T_f)) and reported in Table 5. Plotting the scaled and centered coefficient (Fig. 5–7) revealed that logarithmic transformations were necessary for optimizing the responses. The respective 95% confidence intervals are shown as error bars. Coefficients with 95% confidence intervals including zero were statistically insignificant. Central Composite Face-centred (CCF) quadratic design response factors also indicated at medium, low and high values were indications of the response values of different vendors (Fig. 7). It was observed that different combinations of significant parameters do not drastically affect responses, so that the developed method was considered to be robust.

3.2.6 Stability. The stability of VSN was evaluated in DBS samples by exposing to different conditions (time and temperature) at three QC concentration levels (90, 300 and 1600 ng mL^{-1}) in six replicates. These results were compared with those obtained for 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 h. Auto-sampler stability of VSN was tested by analysis of processed and reconstituted low and high quality control samples, which were stored in the auto-sampler tray for 24 h at 8 °C. Long-term stability was performed by analyzing samples that were stored at −20 °C for 90 days.

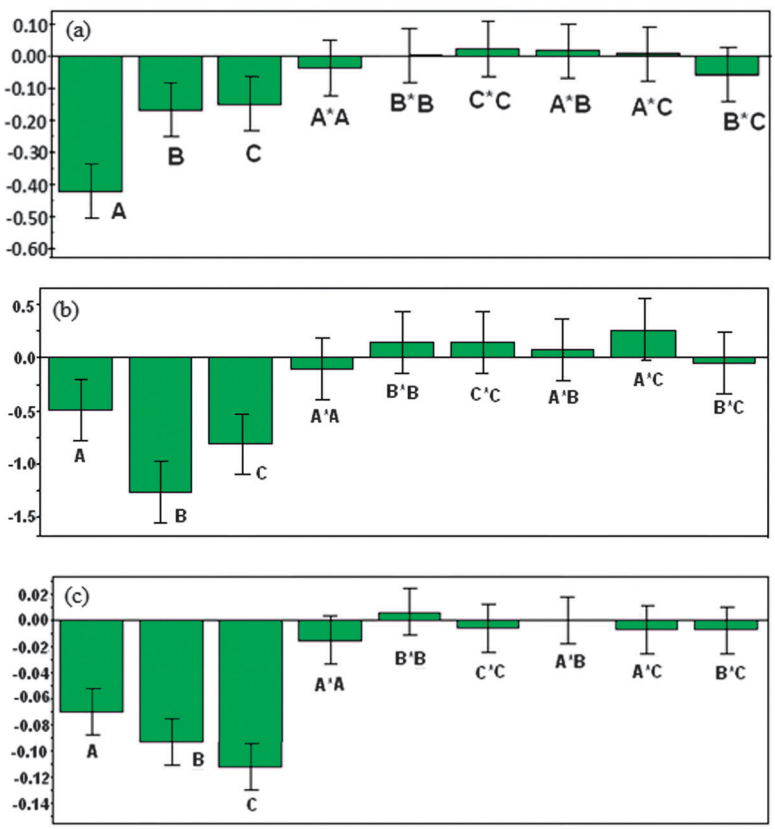

Fig. 5 Scaled and centered coefficient plots of: (a) resolution, R_s; (b) retention time, t_R; (c) tailing factor, T_f.

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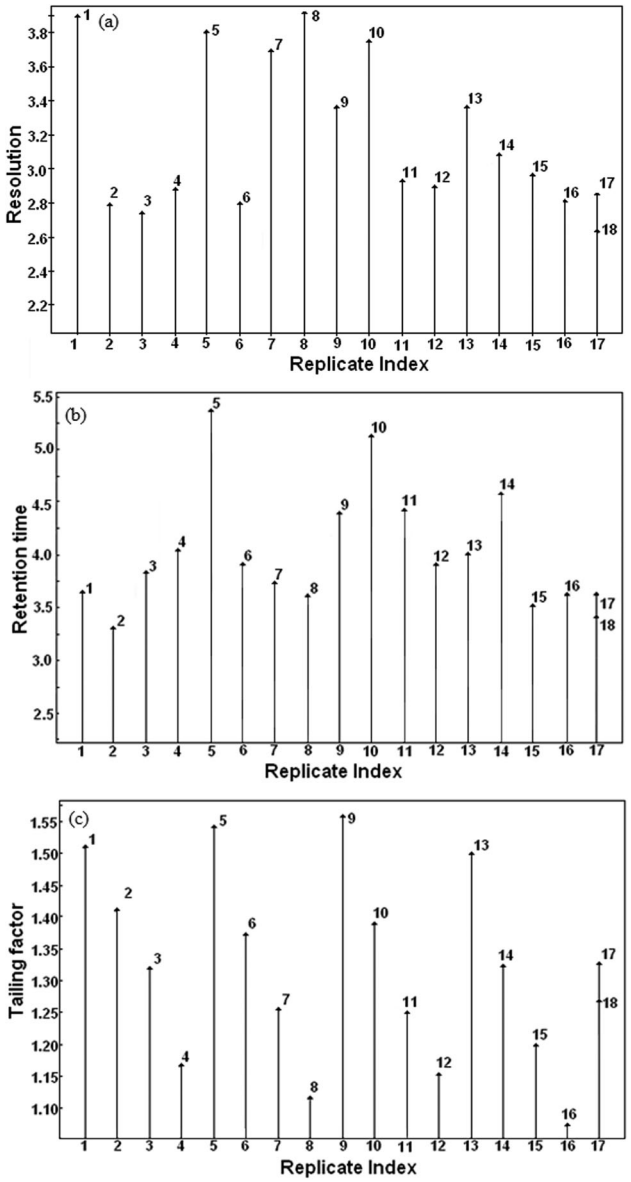

Fig. 6 Plot with experimental number labels of replications for: (a) resolution, R_s; (b) retention time, t_R; (c) tailing factor, T_f.

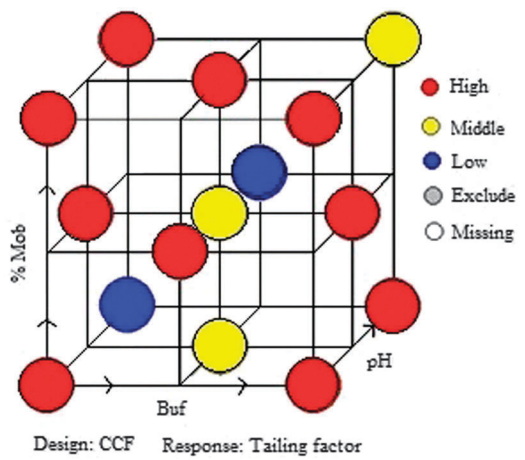

Fig. 7 CCF response factor indication.

Table 6 Short- and long-term stability data of VSN on dried blood spots at three QC levels

	Remaining percentage[a] (mean ± SD)			
Concentration (ng mL^{-1})	Initial	6 h at 25 °C (short-term)	90 days at −20 °C (long-term)	Fourth freeze–thaw cycle
QC-1 90	100.26 ± 4.1	97.11 ± 3.0	102.85 ± 4.4	98.85 ± 4.3
QC-2 300	99.25 ± 3.4	100.48 ± 5.8	97.55 ± 4.8	96.14 ± 2.8
QC-3 1600	99.0 ± 0.9	97.15 ± 6.7	97.14 ± 5.7	99.29 ± 5.8

[a] Remaining percentage = (concentration found)/(concentration added) × 100.

To evaluate the post-preparative stability QC samples were extracted and kept in the auto-sampler (10 °C) for 10 h before the injection. For each concentration and storage condition, six replicates were analyzed in one analytical batch. The concentration of VSN after each storage period was related to the initial concentration as determined for the samples. Table 6 shows short- and long-term stability, four freeze–thaw cycles and 6 h room temperature storage for low and high quality control samples, indicating that VSN was stable in the dried blood sample under the experimental conditions. The results were found to be well within the acceptance limits.

3.2.7 Application to pharmacokinetic in rats. The developed assay method was applied to a pharmacokinetic study after oral administration of VSN to rats at a dose of 30 mg kg^{-1} and means the concentrations in DBS profiles are shown in Fig. 8. The rats were fasted for 12 h and had free access to water before dosing and were further fasted for 2 h after administration. At pre-determined time intervals (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 24 h post administration), 30 μL blood samples were taken from the epicanthic veins and transferred to EDTA-coated tubes. After gentle mixing with anti-coagulant, DBS samples were prepared from each time point. The pharmacokinetic parameters, peak plasma concentration (C_{max}) and the time to C_{max} (t_{max}), elimination half-life ($t_{1/2}$) and area under plasma concentration (AUC) from 0 to infinity ($AUC_{0-\infty}$) were calculated for each subject by the 'Ramkin' software. The pharmacokinetic parameters of VSN are given in Table 7. Therefore, the terminal phase of VSN in the study was well

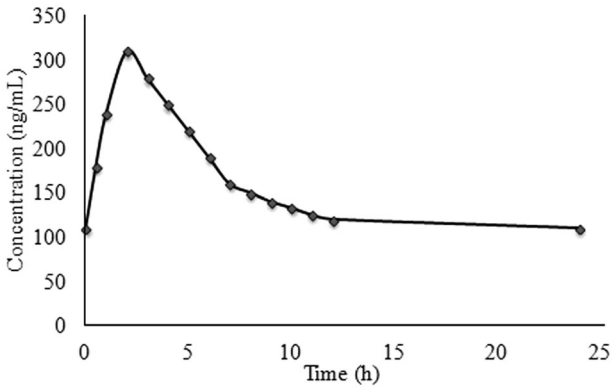

Fig. 8 Concentration–time profile of VSN.

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Table 7 Pharmacokinetic parameters of VSN in rats (n = 6) after oral administration

Parameter	Mean value
Maximum plasma concentration, C_{max} (ng mL^{-1})	310
Time required to reach maximum plasma concentration, T_{max} (h)	1.90
Area under plasma concentration–time curve, $AUC_{0\rightarrow t}$ (ng mL^{-1} h^{-1})	26754.59
Area under plasma concentration–time curve, $AUC_{0\rightarrow \infty}$ (ng mL^{-1} h^{-1})	27689.57
Plasma half life, $t_{1/2}$ (h)	0.3547
Elimination rate constant, K_{el} (h^{-1})	2.04
$AUC_{0\rightarrow t}/AUC_{0\rightarrow \infty}$	0.9662
Mean residence time, MRT (h)	11.2635
Clearance, Cl (L h^{-1})	18.14581

characterized and the analytical assay was able to detect low concentrations at the end of the DBS concentration–time profile. The present developed HPLC assay method could be successfully applied to the determination of VSN in several pharmacokinetic studies conducted in any institution.

4 Conclusions

The developed HPLC method of analysis provided a reliable, reproducible and specific assay for VSN on dried blood spots. The method described here is sensitive enough to detect as low as 30 ng mL^{-1}. The validation method allows quantification of VSN in dried blood samples for the purpose of bioequivalence study, and results were linear from 30 to 2000 ng mL^{-1}. The present assay method assessed extensive validation parameters as per FDA and ICH guidelines.[17,18] The method has shown acceptable precision, accuracy and adequate sensitivity for use in the pharmacokinetic studies and is deemed to be suitable for use in all laboratories equipped with sophisticated or unsophisticated instruments.

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