Simultaneous Quantification of Vincristine and Its Major M1 Metabolite from Dried Blood Spot Samples of Kenyan Pediatric Cancer Patients by UPLC-MS/MS

Lorita Agu[a*], Jodi L. Skiles[b], Andrea R. Masters[b], Jamie L. Renbarger[b], Diana S-L Chow[a]

[a]Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, Texas
[b]Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana

*Corresponding author. Email: loritaikodiya@gmail.com

University of Houston, College of Pharmacy
4849 Calhoun Road, Houston, TX, 77004, USA

Highlights

- Incorporation of formic acid enhanced M1 extraction from human dried blood spots
- Quantification of vincristine and M1 from human dried blood spots
- UPLC-MS/MS method to monitor vincristine and M1 in Kenyan pediatric cancer patients

Abstract

Vincristine (VCR) is an integral part of chemotherapy regimens in the US and in developing countries. There is a paucity of information about its disposition and optimal therapeutic dosing. VCR is preferentially metabolized to its major M1 metabolite by the polymorphic CYP3A5 enzyme, which may be clinically significant as CYP3A5 expression varies across populations. Thus, it is important to monitor both VCR and M1 and characterize their dispositions. A previously developed HPLC-MS/MS method for VCR quantification was not sensitive enough to quantify the M1 metabolite beyond 1 hr. post VCR dose (not published). Establishing a highly sensitive assay is a pre-requisite to simultaneously quantify and monitor VCR and M1, which will enable characterization of drug exposure and dispositions of both analytes in a pediatric cancer population. The addition of formic acid during the extraction process enhanced M1

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extraction from DBS samples. A sensitive, accurate, and precise UPLC-MS/MS assay method for the simultaneous quantification of VCR and M1 from human dried blood spots (DBS) was developed and validated. Chromatographic separation was performed on Inertsil ODS-3 C18 column (5 μm, 3.0 x 150 mm). A gradient elution of mobile phase A (methanol-0.2% formic acid in water, 20:80 v/v) and mobile phase B (methanol-0.2% formic acid in water, 80:20 v/v) was used with a flow rate of 0.4 mL/min and a total run time of 5 min. The analytes were ionized by electrospray ionization in the positive ion mode. The linearity range for both analytes in DBS were 0.6-100 ng/ml for VCR and 0.4-100 ng/ml for M1. The intra- and inter-day accuracies for VCR and M1 were 93.10-117.17% and 95.88-111.21%, respectively. While their intra- and inter-day precisions were 1.05 to 10.11% and 5.78 to 8.91%, respectively. The extraction recovery of VCR from DBS paper was 35.3 – 39.4% and 10.4 – 13.4% for M1, with no carryover observed for both analytes. This is the first analytical method to report the simultaneous quantification of VCR and M1 from human DBS. For the first time, concentrations of M1 from DBS patient samples were obtained beyond 1-hour post VCR dose. The developed method was successfully employed to monitor both compounds and perform pharmacokinetic analysis in a population of Kenyan pediatric cancer patients.

Keywords:

Vincristine; M1 metabolite; Dried Blood Spots; UPLC-MS/MS; Kenyan Pediatric Patients; Cancer

1. Introduction

Vincristine (VCR) is a widely used anticancer agent for the treatment of several malignancies in pediatric oncology. It is an essential part of chemotherapy regimens in resource-limited settings and in the US for its relatively low cost and lack of myelosuppression. However, little information is known about its disposition and optimal therapeutic dosing, and dosing strategies in pediatric patients are still largely empirical [1,2,3,4].

It has been reported that the polymorphic CYP3A5 enzyme metabolizes VCR to its major M1 metabolite more efficiently than the CYP3A4 enzyme [5,6]. This finding maybe clinically significant because CYP3A5 expression varies. Up to 70% of African Americans and 10-15% of

Caucasians express CYP3A5 [5], while 90% of Kenyans express CYP3A5 [2]. Hence, it is crucial to monitor M1 and characterize its disposition in humans to provide an insight into inter-patient variability in VCR metabolism and clearance which will contribute to guide of future rational VCR dosing regimen optimization.

The successful development and validation of an UPLC/MS/MS assay to simultaneously quantify and monitor concentrations of VCR and M1 from dried blood spot (DBS) samples is a pre-requisite for the construction of concentration-time profiles to characterize drug exposures and dispositions of VCR and its M1 metabolite. However, no UPLC/MS/MS assay is available in the literature on how to simultaneously quantify VCR and its M1 metabolite from human DBS samples. Dr. Renbarger and her group were the first to isolate and identify the structures of vincristine metabolites [5,6]. They published an HPLC-tandem mass spectrometry method that quantifies both VCR and its major M1 metabolite, but in plasma [7]. They later developed an HPLC-MS/MS method that quantifies VCR and M1 from DBS samples, but it is not sensitive enough to quantify M1 beyond 1-hr post VCR dose (unpublished data). As a result of this limitation, the complete concentration-time profile of VCR from DBS samples was attainable but not for the M1 metabolite. The purpose of this study was to optimize and validate the HPLC/MS/MS assay developed by Dr. Renbarger's group to simultaneously quantify VCR and its M1 metabolite from DBS samples. The optimized and validated assay has been successfully employed for the quantification of VCR and M1 from DBS samples remotely collected from Kenyan pediatric cancer patients.

2. Materials and Methods

2.1. Chemicals and Materials

Vincristine sulfate (VCR) was purchased from U. S. Pharmacopeia (Rockville, MD, USA). The M1 metabolite, which is not commercially available, was received from Dr. Renbarger at Indiana University School of Medicine (Indianapolis, Indiana, USA). The internal standard, vinorelbine (VRL), Whatman US 903 protein saver card, and formic acid (~98%) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). VRL was used as internal standard because it is structurally similar to VCR. Using a structurally similar compound in lieu of a labeled internal

standard is an accepted practice in bioanalytical labs. LC-MS grade water, acetonitrile, and methanol were purchased from EMD Millipore Corporation (Billerica, MA, USA). Blank human whole blood (Sodium heparin) was purchased from BioreclamationIVT (Westbury, NY, USA).

2.2. Improvement of M1 Extraction

The extraction of M1 from human DBS samples was improved through the addition of 0.2% formic acid in the extraction solvent, water, during the extraction process. The acid additive was added to protonate the M1 chemical structure thus, increasing its solubility in water. Other acids such as acetic acid, hydrochloric acid, and sulfuric acid in water were tested for the improvement of M1 extraction, however, only acidified water using formic acid yielded a positive outcome.

2.3. Chromatographic and Mass Spectrometry Conditions

An ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system was used to analyze VCR and its M1 metabolite. The UPLC system (SCIEX ExionLC™, Framingham, MA, USA) was coupled with an API 5500-Qtrap triple quadrupole mass spectrometer (Applied Biosystem/AB SCIEX, Framingham, MA, USA) equipped with an electron spray ionization (ESI) source in the positive mode. Chromatographic separation was achieved using Inertsil ODS-3 C18 column (5 μm, 3.0 x 150 mm). The mobile phases consisted of methanol: 0.2% formic acid in water (20:80 v/v, mobile phase A) and methanol: 0.2% formic acid in water (80:20 v/v, mobile phase B). Gradient elution was used for separation as follows: 25% B—55% B (0–0.4 min), 55% B—25% B (0.4–3.5 min), 25% B (3.5–5 min). The elution was performed at a flow rate of 0.4 mL/min with an injection volume of 10 μL, while column and autosampler temperatures were set at 25 ^{O}C and 5 ^{O}C, respectively. The quantifications of VCR, M1 metabolite, and VRL internal standard were performed with their respective mass transition pairs presented in Table 1.

Table 1. Mass Spectrometry Settings

Instrument-dependent parameters

Ion Spray Voltage (V)	Temperature (^{O}C)	Collision gas	Curtain gas (psi)	Ion source gas 1 (psi)	Ion source gas 2 (psi)
5500	600	High	20	30	50

Compound-dependent parameters for analytes

Analyte	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
VCR	825.4	807.6	150	7	57	9
M1 metabolite	397.3	337.4	70	7	32	6
VRL	390.3	122.1	50	3	18	6

Parent ion (Q1), Product ion (Q3), declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) for VCR, M1, and VRL, respectively.

2.4. Preparation of Calibration Standards and Quality Control Samples

The standard stock solutions of VCR, M1, and VRL were prepared as follows: VCR at a concentration of 1,000 μg/mL in water and methanol (50:50, v/v). M1 at a concentration of 6.3 μg/mL in a solvent mixture of methanol—0.2% formic acid in water (20:80, v/v). VRL at a concentration of 200 μg/mL in the same solvent mixture used to prepare M1. VCR and VRL stock solutions were stored at -80°C and M1 stock solution was stored at -20°C until used for the preparation of working solutions. Working solutions were made through serial dilutions of these stocks in methanol—0.2% formic acid in water (20:80, v/v) to yield concentrations of 8 (for M1), 12 (for VCR), 20, 100, 200, 500, 1,000, 1,600, and 2,000 ng/ml, respectively. The DBS calibration standards and quality control (QC) samples were prepared separately by spiking the appropriate working solutions (10 μL each of VCR and M1) into 180 μL of whole blood to yield concentrations of 0.4 (for M1), 0.6 (for VCR), 1, 5, 10, 25, 50, 80, and 100 ng/ml. The QC concentrations for VCR and M1 were, a lower limit of quantification (LLOQ) of 0.4 ng/ml for M1 and 0.6 ng/ml for VCR, a low QC of 1 ng/ml, a medium QC of 10 ng/ml, and a high QC of

80 ng/ml. Final VRL internal standard concentration for UPLC-MS/MS analysis was 10 ng/ml. All samples were prepared in the dark due to the sensitivity of VCR to light.

2.5. Sample Preparation and Extraction

Patient DBS samples received were stored in a desiccator away from light at room temperature, in their original amber plastic sample bags containing desiccants. The DBS for calibration standards and QC samples were prepared by adding 20 μL of whole blood spiked with the appropriate concentrations of VCR and M1 to each spot on a Whatman DBS card, with a total of N=5 spots for each concentration level. The spotted DBS samples were allowed to dry overnight at room temperature in the dark. Five discs (6 mm in diameter) were punched out from each DBS samples and placed into 2 mL microcentrifuge tubes. For patient DBS samples, all the spots on the DBS cards at each sample time point were cut out to capture all the areas covered with blood. The cut-out spots were weighed. The punched-out discs from standard samples (N=5) which contained 20 μL of spiked whole blood per spot (~100 μL) were also weighed and used as a reference to obtain the volume of blood in the cut-out spots from patients DBS cards.

To extract VCR and M1, 300 μL of water containing VRL internal standard and 0.2% formic acid were added to each tube, vortexed for 1 minute, and shaken for 1 hour at a speed of 1,100 rpm (Thermo Fischer Compact Digital Microplate Shaker, Waltham, MA, USA). After which, 1 mL of acetonitrile was added to the tubes, vortexed for 30 seconds, and centrifuged at 17,968 x g for 10 minutes at -4ºC. The supernatant was transferred into clean tubes and evaporated to dryness in a stream of air at room temperature. The residual was reconstituted with 50 μL methanol—0.2% formic acid in water (20:80, v/v) for the UPLC-MS/MS analysis. All samples were prepared in the dark away from light by wrapping the samples in aluminum foil and switching the lights off during the entire extraction process. In addition, polypropylene conical inserts containing the reconstituted samples for UPLC-MS/MS analysis were placed in amber glass vials to protect the photosensitive samples from light during the UPLC-MS/MS analysis run.

2.6. Method Validation

The method validation was carried out according to the US FDA Guidelines of Bioanalytical Method Validation: Guidance for Industry [8] for (i) selectivity and specificity (ii) sensitivity and carryover (iii) linearity (iv) accuracy and precision (v) extraction recovery (vi) matrix effect and (vii) stability. Each analytical run included blank samples (no analytes, no IS), zero calibrator samples (blank plus IS), and non-zero calibrator levels covering the quantification range.

2.6.1. Selectivity and Specificity

Selectivity and specificity were assessed by analyzing blank extracts from six individual sources of non-pooled blank human whole blood spotted on DBS paper. To ensure selectivity and specificity, there should be no interference with signals greater than 20% of the LLOQ response of the analytes at the retention times of VCR and M1. Also, there should be no interference with signals greater than 5% of the IS response at the retention time of the VRL IS.

2.6.2. Sensitivity and Carryover

Sensitivity was evaluated by analyzing six replicates of spiked LLOQ DBS samples. Accuracy and precision should be within 20% of the nominal concentration of the LLOQ. The LLOQ was determined as the concentration producing a peak response greater than or equal to 5 times the response of DBS blank at the same retention time. Carryover was assessed by injecting 5 samples at the high QC concentration followed by three blank injections. Carryover should not exceed 20% of LLOQ and 5% of IS.

2.6.3. Linearity

Linear calibration curves were constructed by plotting the analyte/IS peak area ratios versus the analyte nominal concentrations. Calibration curves were fitted by linear regression using a weighting factor of 1/x for both VCR and M1.

2.6.4. Accuracy and Precision

Accuracy and precision were determined using four QC samples—LLOQ (0.4 ng/ml for M1 and 0.6 ng/ml for VCR), low QC (1 ng/ml), medium QC (10 ng/ml), and high QC (80 ng/ml). The stock solution used to prepare the QC samples was separate from the stock solution used to prepare calibration curve samples. Intra- and inter-day accuracy and precision were established

by analyzing six replicates of the four QC sample levels on three independent days. Accuracy was expressed as a percentage of the ratio of calculated to nominal concentrations while precision was expressed by the % of coefficient of variation (CV). The acceptance criteria for accuracy is ± 15% of nominal concentrations except at LLOQ where ± 20% of nominal concentration is permitted. The acceptance criteria for precision is ± 15% CV while at LLOQ ± 20% CV is allowed.

2.6.5. Recovery and Matrix effects

Recovery and matrix effects were determined at three QC levels (low QC, medium QC, and high QC). Recovery was determined by comparing the peak area ratios of analytes from pre-extracted DBS samples (analytes in blood and spotted on DBS paper before extraction) to analytes from post extracted DBS samples (analytes added after extraction of blank blood on DBS paper). Matrix effects was determined by comparing the peak area ratios of analytes from post extracted DBS samples to analytes from neat standard solution.

2.6.6. Stability and Hematocrit Effects

The stability of VCR and M1 in DBS paper and in processed samples were evaluated under certain conditions. The samples were hand carried home on a commercial flight (i.e., at room temperature) in the passenger cabin, hence, there was no issue with significant swings in temperature variability. Once samples arrived at the lab, they remained at room temperature. Stability of the analytes in DBS paper was assessed at 25°C for 4 months to determine the storage stability. The stability of processed samples was carried out by re-injecting the samples after storage in the autosampler at 5°C for 24 hours. Stability studies were performed at two QC levels (low QC and high QC) and compared to measurements from freshly prepared samples. The accuracy at each level should be within ± 15% of the initial concentration. Previous work from Dr. Renbarger and group showed hematocrit effects at levels 30 and 45 (unpublished).

2.7. Method Application

This method was used to quantify VCR and M1 from DBS samples of Kenyan pediatric cancer patients involved in a phase 1 clinical study (IRB protocol # 1307011716R004). In this paper, concentration-time profiles from two representative subjects were used to demonstrate the

application of this established and validated method. Patient 1 was a 5-year-old female with nephroblastoma (NEPHRO) who received 2.5 mg/m^2 VCR dose. Patient 2 was a 14-year-old male with acute lymphoblastic leukemia (ALL) who received 2.0 mg/m^2 VCR dose. VCR dose was administered through intravenous push. Sampling time points for patient 1 were at 0.5, 1, 18.25, 19.25, 41.75, and 66.67 hours post VCR dose while those for patient 2 were at 2.5, 3, 26.4, and 26.9 hours post VCR dose. Collection of sampling time points depended on the feasibility and duration of patient stay for care in the hospital.

3. Results

The structures of VCR, M1 metabolite, and VRL internal standard are shown in Figure 1.

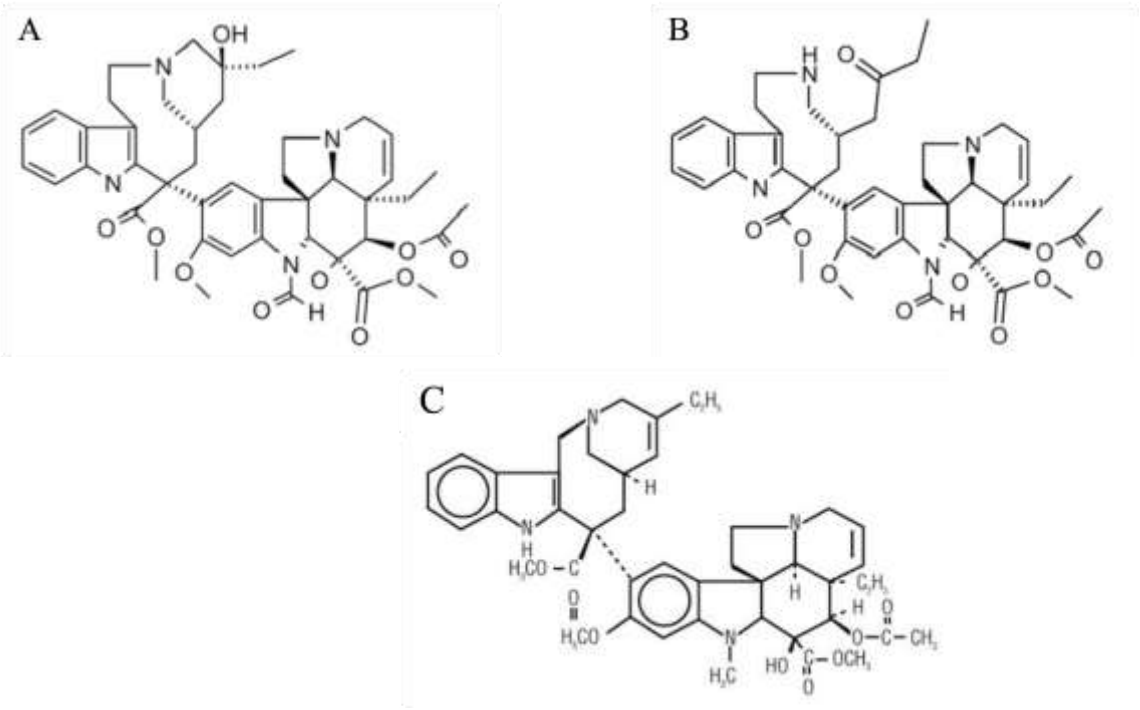


Figure 1. Structures of VCR (A), M1 metabolite (B), and VRL internal standard (C). (Dennison et al. 2008. Therapeutic Drug Monitoring[7]; Vinorelbine FDA Drug Label[15]).

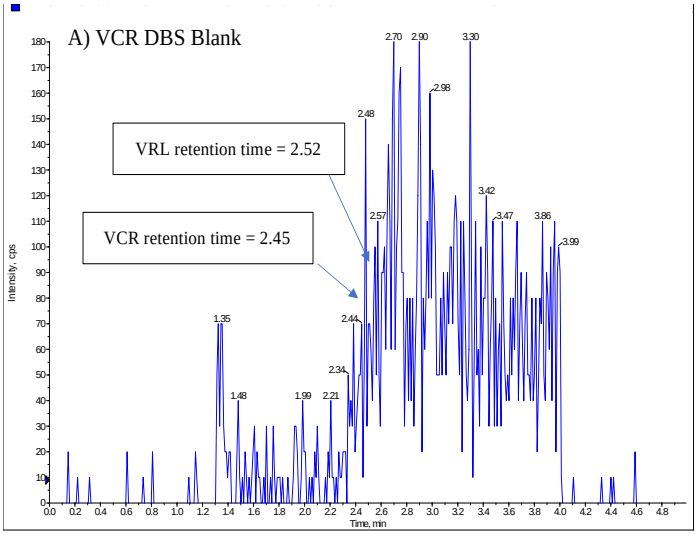
3.1. Selectivity and Specificity

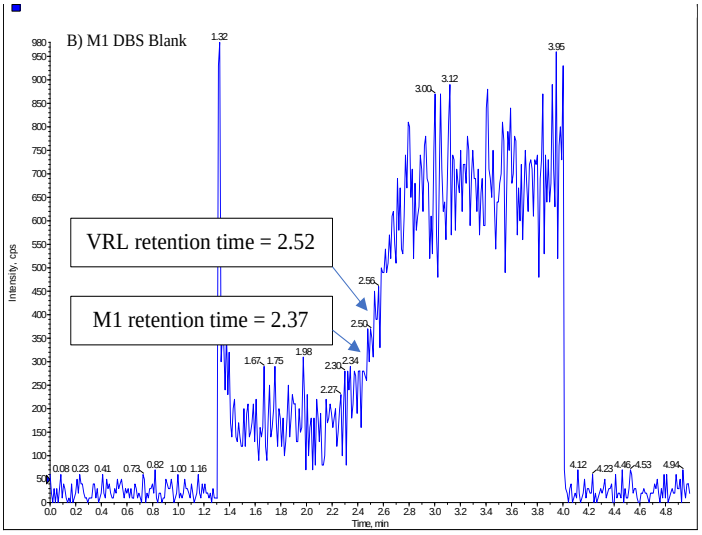
Extracts from blank whole blood on DBS paper were analyzed to evaluate the degree of interference from the DBS blank sample at the peak areas of VCR, M1 and VRL IS. No

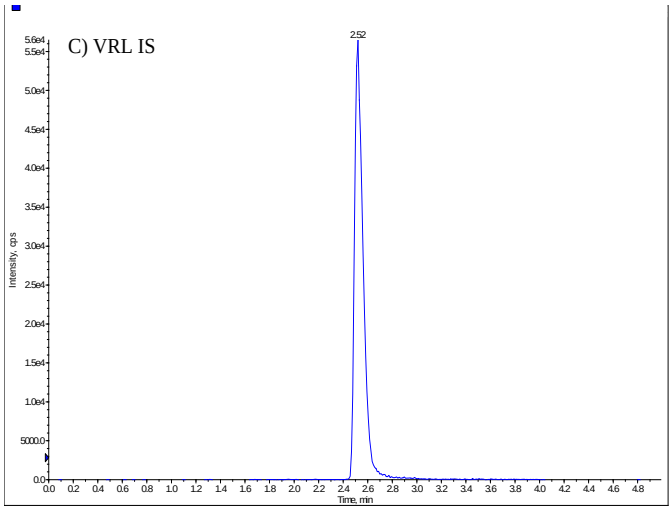
interference > 20% of VCR and M1 peak area at LLOQ and nor > 5% VRL internal standard (IS) peak area was observed (Figure 2).

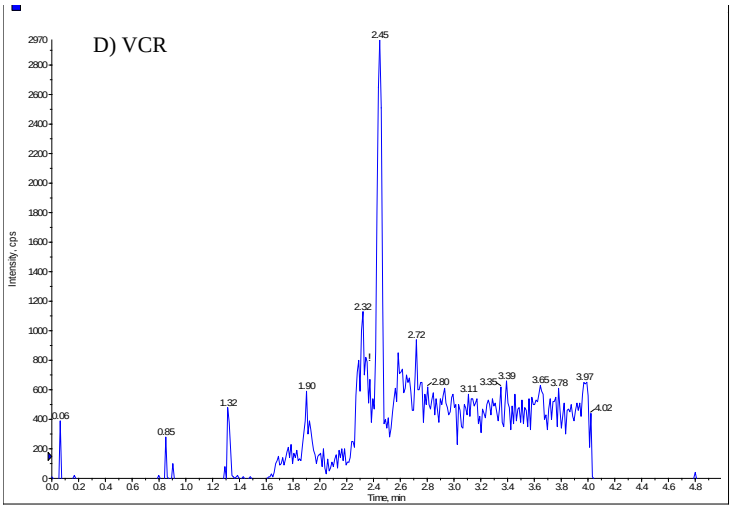
3.2. Sensitivity and Carryover

The LLOQ for VCR and M1 were 0.6 ng/mL and 0.4 ng/mL, respectively, yielding a signal to noise ratio > 5 for both analytes. The chromatograms for VCR and M1 at LLOQ are shown in Figure 2. No carryover > 20% of the LLOQ response for both analytes and no carry over > 5% of the response for the VRL IS were observed in the blank injections following 5 injections of samples at the high QC concentration level.


A) VCR DBS Blank
VRL retention time = 2.52
VCR retention time = 2.45
Intensity, cps
Time, min


B) M1 DBS Blank
VRL retention time = 2.52
M1 retention time = 2.37
Intensity, cps
Time, min


C) VRL IS
Intensity, cps
Time, min


D) VCR
Intensity, cps
Time, min

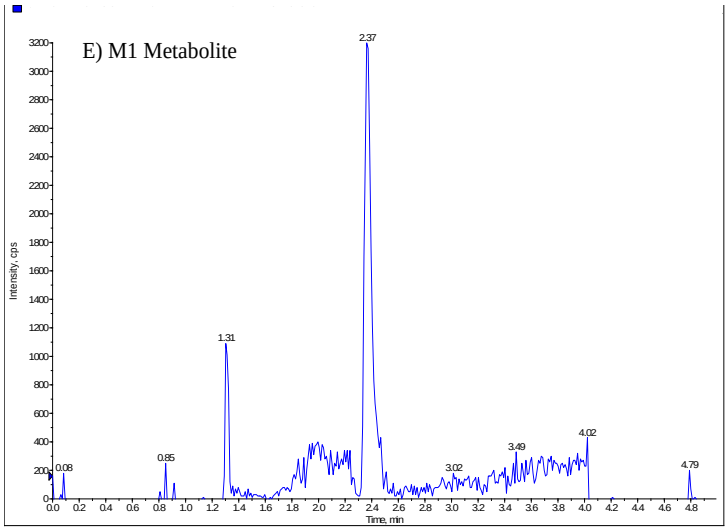

E) M1 Metabolite
Intensity, cps
Time, min

Figure 2. Representative Chromatograms showing DBS blanks of VCR and M1 (A, B) VRL IS (C) VCR LLOQ (D) M1 LLOQ (E). The retention times for VRL IS, VCR, and M1, are 2.52, 2.45, and 2.37 min, respectively.

3.3. Linearity

The calibration curves for both analytes in DBS, which were used for analysis, were linear over the concentration range of 0.6-100 ng/ml for VCR and 0.4-100 ng/ml for M1 with coefficients of correlation of at least 0.99 (Figure 3).

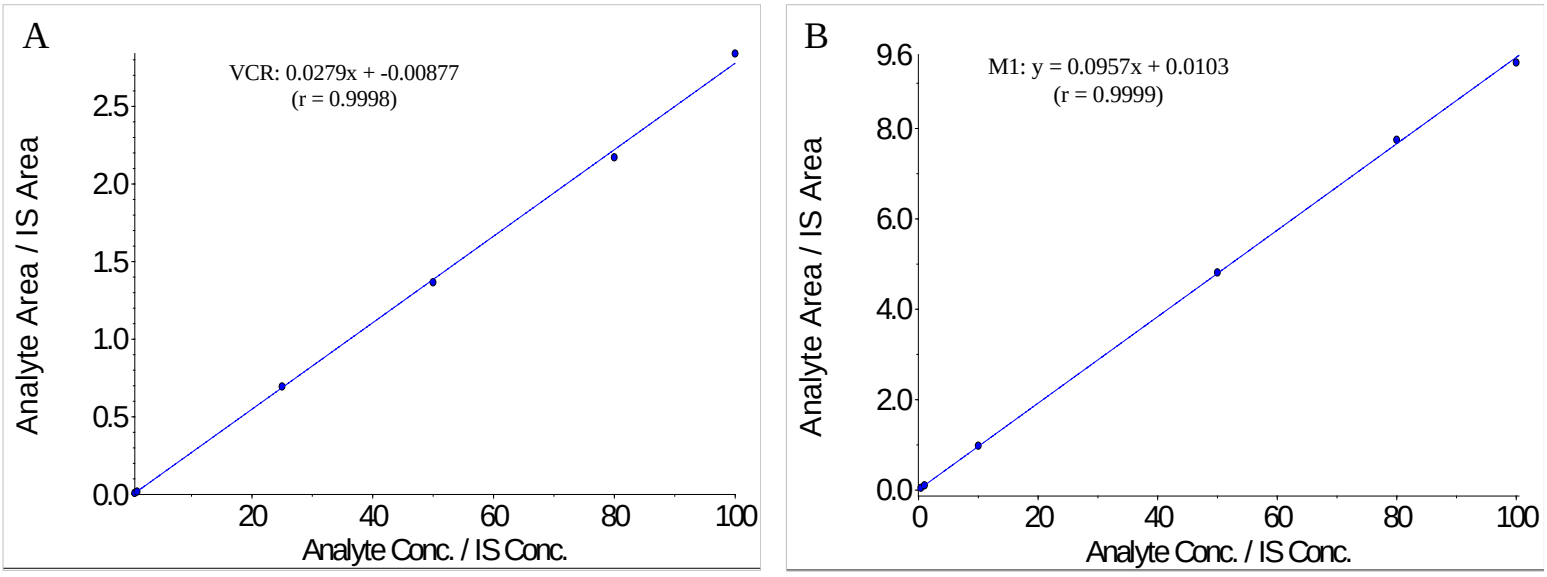


Figure 3. Calibration Curves of VCR (A) and M1 (B) from DBS Samples

3.4. Accuracy and Precision

The intra-day accuracy and precision values for VCR ranged from 93.35 to 110.17% and 3.60 to 8.59%, respectively, while the intra-day accuracy and precision values for M1 ranged from 93.10 to 117.17% and 1.05 to 10.11%, respectively. The inter-day accuracy and precision values for VCR ranged from 95.88 to 108.54% and 6.63 to 8.49%, respectively, and those of M1 ranged from 99.66 to 111.21% and 5.78 to 8.91%, respectively. The values of accuracy and precision for both analytes were within the 15% acceptable limit and 20% limit for LLOQ (Table 2).

Table 2. Intra- and Inter-day Accuracy and Precision of VCR and M1 in DBS Samples

Intra-day (N=6)							**Inter-day (N=18)**	
VCR								
QC level	**Day 1**		**Day 2**		**Day 3**			
ng/ml	**Accuracy (%)**	**Precision (%)**	**Accuracy (%)**	**Precision (%)**	**Accuracy (%)**	**Precision (%)**	**1 Accuracy (%)**	**Precision (%)**
0.6	110.17	5.62	106.85	8.59	108.62	6.32	108.54	6.63
1	109.33	3.78	99.08	6.87	102.20	7.84	103.54	7.29
10	97.83	7.27	96.45	8.23	93.35	6.82	95.88	7.30
80	105.93	8.14	96.15	7.62	93.63	3.60	98.57	8.49
M1 Metabolite								
0.4	116.50	1.43	99.95	10.11	117.17	1.91	111.21	8.91
1	111.17	1.05	97.85	6.19	102.48	8.21	103.83	7.72
10	100.33	6.06	99.73	5.48	106.50	3.97	102.19	5.78
80	93.10	4.79	94.87	1.98	111.00	2.93	99.66	8.91

3.5. Recovery and Matrix effects

The extraction recovery of VCR from DBS paper was 35.3 – 39.4% and 10.4 – 13.4% for M1. The matrix effects of VCR and M1 in DBS paper were 42.0 – 63.4% and 47.2 – 64.4%, respectively. The mean (± SD) of extraction recovery and matrix effects are presented in Table 3. Regardless of the low values obtained for extraction recovery, possibly due to ion suppression as seen from the matrix effects, an LLOQ of 0.6 ng/ml for VCR and 0.4 ng/ml for M1 were reached with acceptable accuracy and precision as shown in Table 2.

Table 3. Recovery & Matrix Effects (Mean ± SD) of VCR & M1 from DBS Samples

	Recovery (%)		**Matrix Effects (%)**	
QC level (N = 3)	**VCR**	**M1**	**VCR**	**M1**
1 ng/ml	39.45 ± 3.41	13.41 ± 1.47	63.45 ± 7.63	58.8 ± 4.12
10 ng/ml	38.98 ± 3.43	10.47 ± 0.30	42.08 ± 3.29	47.21 ± 1.95
80 ng/ml	35.34 ± 3.20	12.03 ± 0.66	55.34 ± 5.05	64.45 ± 3.78

3.6. Stability and Hematocrit Effects

The stability results for VCR and M1 are presented in Table 4. VCR was stable for up to 4 months at 25 ℃ in DBS samples stored in the dark. M1 was stable for up to 4 months at 25 ℃ at the low QC concentration but not at the high QC concentration. However, Dr. Renberger and her group found that both VCR and M1 in DBS samples were stable for up to 5 months at 25 ℃ with accuracy ranging from 86-96% for VCR and 89-93% for M1 (not published). Processed samples were stable up to 24 hr in the autosampler at 5ºC and hence can be analyzed overnight (within 24 hr). According to work from Dr. Renberger and her group, the accuracy for VCR and M1 at HCT of 30 and 45 ranged from 83-100 % for VCR and 82-100% for M1, respectively (not published; see Table S1 in supplemental data).

Table 5. DBS Storage and Processed Sample Stability of VCR and M1

4-Months Storage (Mean ± SD: % CV)			
QC level (N = 3)	**Temperature**	**VCR Stability (%)**	**M1 Stability (%)**
1 ng/ml	25 ℃	90.67 ± 4.57 (5.04 %)	92.67 ± 2.95 (3.18 %)
80 ng/ml	25 ℃	97.57± 0.98 (1.01 %)	15.20 ± 1.91 (12.53 %)
Processed samples/autosampler for 24 hrs.			
1 ng/ml	5℃	96.43 ± 6.67 (6.91 %)	105.11 ± 1.79 (1.71 %)
80 ng/ml	5℃	101.54 ± 2.29 (2.25 %)	112.33 ± 0.84 (0.75 %)

3.7. Method Application

The described LC-MS/MS method was successfully used to quantify VCR and M1 from DBS samples of Kenyan pediatric cancer patients. Figure 4 shows the concentration-time profiles of two representative subjects whose profiles were obtained using the described method.

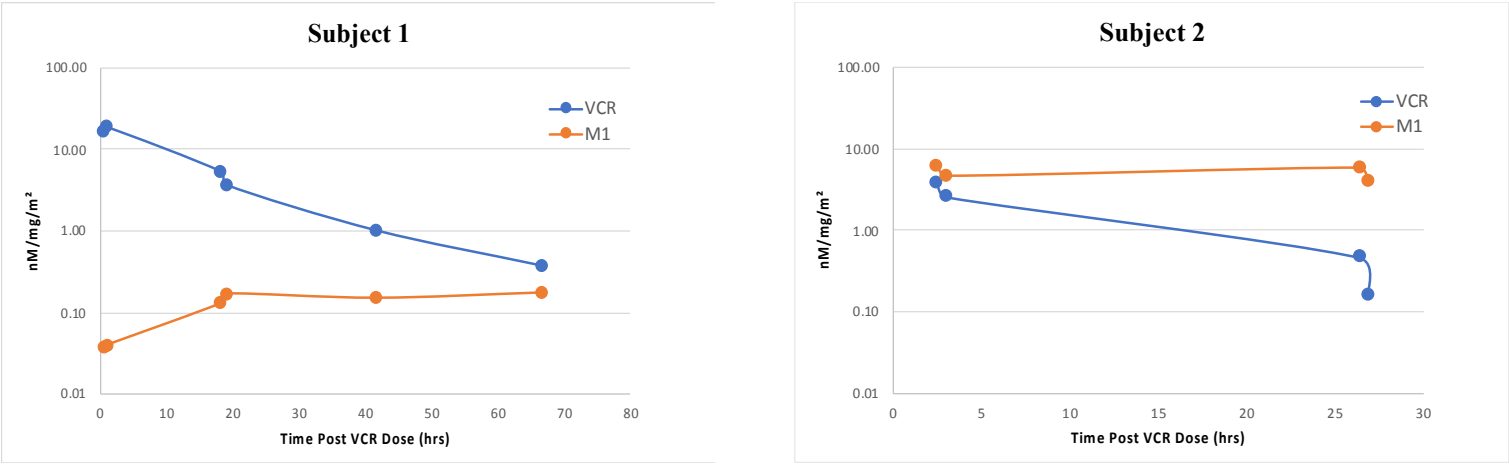


Figure 4. DBS Concentration-Time Profiles of VCR & M1 from 2 representative Kenyan Pediatric Cancer Patients. Subject 1 (NEPHRO patient) received 2.5 mg/m^2 VCR dose and subject 2 (ALL patient) received 2.0 mg/m^2 VCR dose.

4. Discussion

VCR is a widely used anticancer agent and an integral part of chemotherapy regimens for treatments of several malignancies in pediatric oncology. Notwithstanding, there is still a paucity of information about VCR disposition and optimal therapeutic dosing. The knowledge that VCR is preferentially metabolized to its M1 major metabolite by the polymorphic CYP3A5 enzyme, whose expression varies across populations, indicates that it is vital to characterize the disposition of M1 in humans to provide an insight into the inter-patient variability in VCR metabolism and clearance; such pharmacokinetic characterization of VCR and M1 will be helpful and essential for future dosing regimen optimization. In order to successfully delineate the disposition of M1, it is imperative to monitor and quantify M1 using a sensitive and robust analytical assay.

An accurate and precise method on how to simultaneously quantify both VCR and its M1 metabolite from DBS paper is described. There are several publications on the quantification of vincristine in human plasma. However, only one publication quantified both VCR and its M1 metabolite in human plasma [7]. Although plasma is the conventional sampling method, DBS sampling offers a number of advantages over the traditional plasma sampling method. The minimal volume of blood required and less invasive sampling from patients makes the DBS method an attractive option especially for vulnerable populations such as pediatric populations where there are limitations on how much blood can be safely collected from patients. In addition, the ease of sample collection, storage, and transport of DBS samples makes DBS method useful and feasible in remote settings where medical limitation or lack of resources prevail [9,10,11,12,13]. There is only one publication on the quantification of VCR from DBS in the literature [14]. However, this published method did not quantify the M1 major metabolite of VCR compared to our method which simultaneously measures both compounds. Our method is also more sensitive than the previously published method which had an LLOQ of 1 ng/mL for VCR compared to an LLOQ of 0.6 ng/mL in our described method.

To the best of our knowledge, this is the first report to simultaneously quantify VCR and its M1 metabolite from DBS samples. Dr. Renbarger and her group developed an HPLC-MS/MS method that quantifies VCR and M1 from DBS samples, but it was not sensitive enough to quantify M1 beyond 1 hr. post VCR dose (not published). This resulted in the inability to fully

construct concentration-time profiles to pharmacokinetically characterize drug exposures and dispositions of VCR and its M1 metabolite. Hence, it was crucial to optimize and validate the HPLC/MS/MS assay developed by Dr. Renbarger's group to simultaneously quantify VCR and M1 from DBS samples. The incorporation of formic acid additive during the extraction process enhanced the extraction of M1 from DBS samples and for the first time, concentrations of M1 from DBS patient samples were obtained beyond 1-hour post VCR dose. In addition to the ability of measuring both analytes concurrently in this reported method, the analytical run time was only 5 minutes compared to the 15 minutes run time in the old method. Hence, a method that is sensitive, selective, specific, accurate, and precise for VCR and M1 quantifications from DBS samples has been developed. The linear range of this method for VCR is 0.6-100 ng/mL and 0.4-100 ng/mL for M1. The pharmacokinetic application of this method confirms that VCR and its M1 metabolite can be monitored and quantified from DBS samples of pediatric cancer patients. In comparison of VCR plasma and DBS concentrations, studies in the literature[16, 17, 18, 19, 20] show that VCR plasma $AUC_{0\text{-}Infinity}$ values ranged from 65.1 - 182.6 ng*hr./ml compared to $AUC_{0\text{-}Infinity}$ of 330.25 ng*hr./ml obtained from our DBS study. The larger AUC value in our DBS study compared to plasma may suggest that VCR accumulates or binds to red blood cells which could result to less VCR concentration during plasma retrieval from whole blood. More research is needed in this area. Lastly, with this method, concentration-time profiles were established, which would enable characterization of drug exposure and dispositions of VCR and M1 in a population of pediatric cancer patients by pharmacokinetic modeling for future simulation modeling and potential regimen modification.

5. Conclusion

A sensitive, specific, accurate, and precise UPLC-MS/MS assay method was developed and validated for the simultaneous quantification of VCR and its M1 metabolite from DBS samples. This is the first analytical method to report the concurrent quantification of VCR and M1 from DBS samples. The developed method was successfully employed to monitor both compounds and perform pharmacokinetic analysis in Kenyan pediatric cancer patients.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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