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Research Article

ADVANCEMENT OF DRIED BLOOD SPOT TECHNIQUE WITH REMDESIVIR BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION IN HUMAN BLOOD AS PER ICH M10 GUIDELINE

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ABSTRACT

Objective: The objective of the study was to develop and validate a simple, accurate, and sensitive dried blood spot (DBS) assisted liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the determination of remdesivir in human blood using remdesivir D5 as internal standard as per ICH M10 guideline.

Methods: For quantification, an electrospray ionization source with multiple reaction monitoring was employed on a Thermo Fisher Scientific Accela high-performance liquid chromatography (HPLC) coupled with a TSQ ENDURA mass spectrometer. Waters Symmetry C18 column, used in combination with a highly organic acidified mobile phase, provided a prominent and consistent response with a run time of 3 min. The DBS technique was further refined using Whatman 903 DBS card to extract Remdesivir in acidified methanol.

Results: Method validation was conducted in accordance with ICH M10 guidelines. This method demonstrated excellent performance, with within-run and between-run precision below 7% and QC sample accuracy ranging from 95% to 109%.

Conclusion: Validated calibration range of 50–5000 ng/mL is well-suited for human clinical or therapeutic drug monitoring studies, particularly given Cmax of approximately 2229 ng/mL observed in human.

Keywords: Remdesivir, Dried blood spot, Bioanalytical method validation, Bioanalysis, Clinical study, ICHM10, LC-MS/MS.

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INTRODUCTION

The limitations of traditional blood sampling methods have motivated the development and adoption of the dried blood spot (DBS) technique, which offers several advantages, including minimally invasive sampling, ease of transport, cost-effective storage, and broad applicability in clinical research settings [1,2]. In the DBS technique, a drop of blood will be applied to a specialized filter paper as part of sample processing. This method facilitates the extraction of target components for quantitative bioanalysis [3]. This novel approach offers several benefits, including minimal blood withdrawal, simplicity, feasibility of self- or unskilled sampling, and reduced resource requirements [4,5]. Furthermore, DBS cards can be considered non-infectious, as viruses and other microbes become inactive upon drying. As shown in Fig. 1, DBS can serve as a substitute for conventional methods, offering significant advantages at every stage, from sample collection to analysis.

DBS enables minimally invasive blood collection, making it particularly valuable for research involving infants and young children. This advantage has garnered significant attention in pediatric studies [5,6]. In addition, DBS can be utilized in household settings to monitor patient compliance, as it allows for simple and convenient sample collection [7]. DBS can be effectively used for disease surveillance and screening in resource-limited settings, as it simplifies both sample collection and transportation [3,8]. With these numerous advantages, we propose the development of a Remdesivir-centric DBS bioanalytical method for use in clinical studies and therapeutic monitoring. However, DBS method is inherently complex and requires careful consideration of multiple factors, including drug chemistry, filter paper characteristics, intended application, and population variability.

COVID-19 is a condition caused by the SARS-CoV-2 virus which reported worldwide [5]. In October 2022, remdesivir was introduced as a broad-spectrum small-molecule antiviral drug with efficacy against RNA viruses [9]. Once administered in the body, remdesivir is metabolized into remdesivir triphosphate, which mimics the primary building block of viral RNA but disrupts the viral replication cycle, thereby inhibiting its progression [10]. In our research work, sensitive and selective DBS method was developed using an electrospray ionization source with multiple reaction monitoring (MRM) on Thermo TSQ ENDURA for pharmacokinetic studies and therapeutic drug monitoring (TDM) [11]. As Cmax observed as 2229 ng/mL, so DBS method was validated following the ICH M10 guideline with a linearity range of 50–5000 ng/mL [12]. These validation results indicate that this method will be suitable for both patient care and regulatory submission studies [13]. As per one research article, researchers have developed a Volumetric Absorptive Microsampling (VAM) method for remdesivir; however, VAM cannot be considered an alternative to or equivalent to DBS. VAM is preferable only in specific situations where precise blood volume is critical for analysis, hematocrit correction is mandatory for accurate quantification. Overall, DBS remains a simpler, cost-effective and widely accepted method, making it more practical than VAM in many bioanalytical and clinical applications [14]. Some researchers also used remdesivir as an Internal standard (IS) for their assay [15]. According to another research paper, a paper spray method for remdesivir in plasma was developed, where researchers aliquoted human plasma onto a plastic disc with paper, allowing it to dry for an extended period before bioanalysis. However, in our developed method, we used whole blood directly instead of plasma. Compared to this our approach offers significant advantages, including reduced blood volume requirements significantly along with removal of intermediate steps for plasma separation [16].

METHODS

Material, chemicals, and reagents

Remdesivir (Purity 99.74%) and remdesivir D5 (99.8% D atom isotopic enrichment) working standard, which confirms its identification by mass and nuclear magnetic resonance, obtained from Vivan Life Sciences India. Caffeine (Purity 99.95%), ibuprofen (Purity 99.05%), diclofenac (99.94% Purity), paracetamol (Purity 99.42%), ondansetron (Purity 99.94%), and ranitidine (Purity 99.67%) were required for specificity obtained from the same manufacturer. Cytiva's Whatman 903 DBS with 17 mm mean spot diameter and 9 s/100 µL mean blood absorption time, as shown in Fig. 2 obtained from Vital Science, India.

High-performance liquid chromatography (HPLC)-grade Acetonitrile and Methanol (J.T. Baker), Ammonium Acetate, Ammonium Formate and Formic Acid (Merck), and Milli-Q water were used.

Human blood

Human blood was collected at Clinical Lab, Navi Mumbai, India, following approval from Ethicare Ethics Committee, India (ECR/224/Indt/MH/2015/RR-21), for a clinical blank study (Protocol PCLPL-001-24). This study was specifically designed for the collection of human blood and other biological matrices for research purposes only. A trained phlebotomist collected blood from healthy human subjects who met the inclusion criteria and successfully passed the screening procedure. Blood samples from multiple donors were collected in dipotassium ethylenediaminetetraacetic acid (K_2EDTA) anticoagulant tubes and stored at 2–8°C to maintain blood integrity.

Instrument and equipment

Extraction from human biological matrices often retains a significant amount of matrix components, even after purification. Therefore, liquid chromatography tandem mass spectrometry (LC-MS/MS) with Electrospray Ionization (ESI) source in MRM mode with the capability to selectively quantify the analyte was chosen as an analytical technique due to its high sensitivity and selectivity. During this research, Accela model HPLC system of Thermo Fisher Scientific, coupled with a TSQ ENDURA mass spectrometer model of Thermo Fisher Scientific was used for analysis. Data acquisition and processing were performed using LCquan 3.0 software. Nitrogen

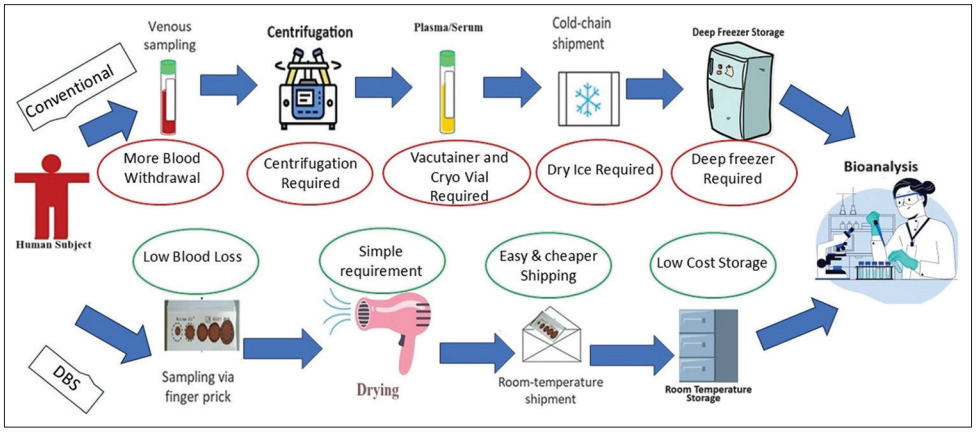

Fig. 1: Comparison of dried blood spot and conventional plasma method

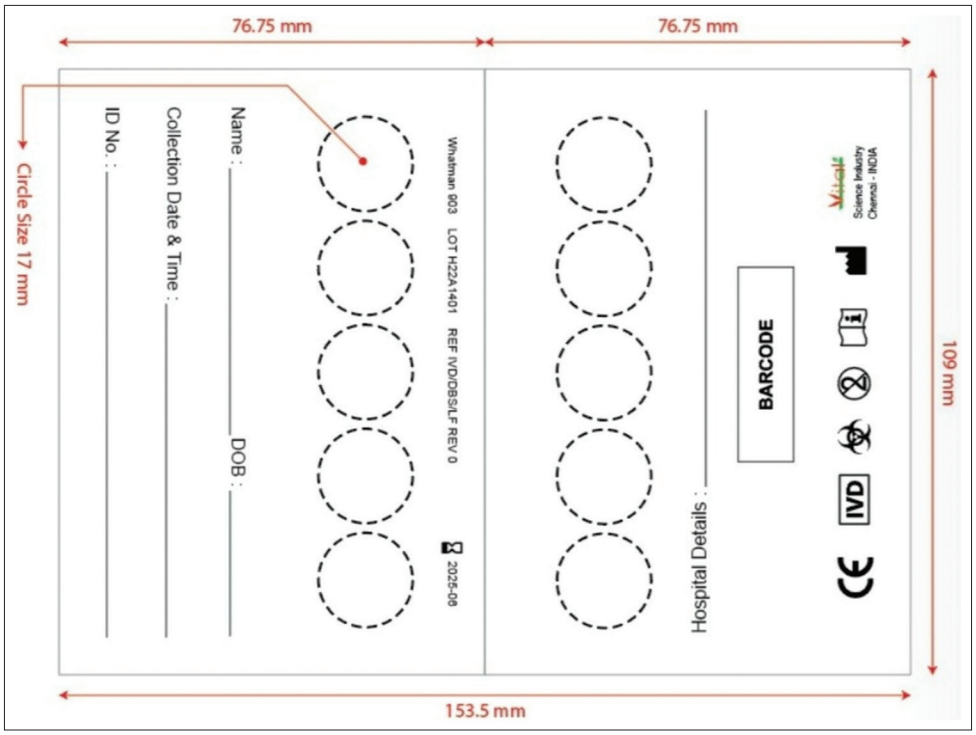

Fig. 2: Whatman 903 dried blood spot paper

Evaporator of N_2 Fastvap model from PEI Analytics, centrifuge of Electrotecnik model from REMI, and Vortex shaker from Praveen Scientific Corporation were used.

Stock preparation (analyte and IS)
As per requirements and the ICH M10 guideline, separate and fresh stock were prepared during stability assessment for calibration curve (CC) and quality control (QC) sample preparation. Remdesivir and Remdesivir D5 standard were dissolved in methanol to prepare a stock of 1 µg/mL nominal concentration.

Preparation working intermediate dilutions
CC and QC intermediate working dilutions were prepared from the main stock solution using a methanol: water (80:20, v/v) diluent. The IS dilution was prepared from Remdesivir D5 stock solution to achieve a final concentration of 1000 ng/mL, which was optimized based on the required response in extracted samples.

Human blood spiked sample preparation
To prepare blank spiked human blood samples, methanol: water (80:20, v/v) was added to blank human blood to create a zero standard (blank) and Blank + IS sample. For non-zero standards, working dilutions of Remdesivir were spiked into blank human blood to achieve a CC standard range of 50–5000 ng/mL. To cover the Cmax of Remdesivir in human studies, Table 1 was considered as a guide while targeting the spiking concentration in human blood.

As per requirements of the ICH M10 guideline, individual aliquots of each standard were prepared in polypropylene tubes to ensure consistency and stability. Spiking ratio of QC working solutions was aligned with that of CC standards, to achieve QC concentrations from 50 to 4000 ng/mL in human blood.

Method development
Development of a DBS bioanalytical method using LC-MS/MS required additional steps beyond traditional HPLC-based analytical method development. Mass tuning involving mass parameter adjustment, extraction procedure optimization involving extraction trials, and development of DBS-specific techniques was to enhance sensitivity, selectivity, and overall method robustness [17].

Optimization of mass spectrometric conditions
Mass spectrometric parameters were optimized on LC-MS/MS TSQ ENDURA system using an ESI source. For mass source tuning, a 100 ng/mL solution of remdesivir and remdesivir D5 was prepared in methanol. A continuous syringe infusion pump was used to introduce this solution directly into the ESI source. The objective was to achieve an optimal response in quadrupole-1 (Q1) and quadrupole-3 (Q3) by systematically adjusting compound and source-dependent parameters.

Table 1: Targeted CC and QC concentrations

Level	Criteria	Concentrations
CC-1	LLOQ	50 ng/mL
CC-2	×2 of LLOQ	100 ng/mL
CC-3	5% of ULOQ	250 ng/mL
CC-4	15% of ULOQ	750 ng/mL
CC-5	50% of ULOQ	2500 ng/mL
CC-6	75% of ULOQ	3750 ng/mL
CC-7	90% of ULOQ	4500 ng/mL
CC-8	ULOQ	5000 ng/mL
LLOQ QC	100% of LLOQ	50 ng/mL
LQC	×3 of LLOQ	150 ng/mL
MQC	50% of ULOQ	2500 ng/mL
HQC	80% of ULOQ	4000 ng/mL

CC: Calibration curve, QC: Quality control, LLOQ: Lowest limit of quantification, ULOQ: Upper limit of quantification, HQC: High quality control, MQC: Middle quality control, LQC: Lower quality control

Optimization of chromatographic condition
Chromatographic optimization was performed through systematic, science-based trials, considering polarity and pKa (negative logarithm of acid dissociation constant) of the target molecule [18,19]. Given the polar nature of remdesivir, different C18 columns and various mobile phase compositions ranging from non-polar to low-polar solvents were evaluated under different trials. During initial trials, a Hypersil Gold C18 and Hypurity C18 column was tested. However, a Waters Symmetry C18 column (300Å, 5 µm, 3.9 mm × 150 mm) with an acidified acetonitrile: 10 mM ammonium acetate mobile phase was selected as the final condition due to its ability to produce well-resolved and stable chromatographic peaks.

Optimization of DBS extraction method
During DBS technique development, various DBS papers from multiple sources were evaluated. A hole punch plier (ticket punch) with a 10 mm diameter cut was used to optimize DBS technique, with a standardized blood spotting volume of 0.2 mL. After drying, several extraction trials were conducted to efficiently extract Remdesivir from DBS paper. In the optimized extraction procedure, a 10 mm DBS paper disc was vortexed and centrifuged in 1 mL of acidified methanol. Later, this supernatant was evaporated at 40°C under 10 psi air pressure using an N_2 Fastvap Nitrogen Evaporator. The dried residue was then reconstituted in an optimized mobile phase consisting of methanol:10 mM ammonium acetate (pH 3) in a 95:5% (v/v) ratio before LC-MS/MS analysis. To minimize contamination from the hole punch, calibration standards and QC samples were punched from low to high concentrations. In addition, the punch was rinsed between samples using 100% methanol with a blank paper punch to prevent carryover.

Hematocrit effects
Hematocrit is a critical factor that can significantly impact drug quantification in DBS analysis [20-22]. Patients with higher hematocrit levels tend to have more viscous blood, resulting in smaller blood spots, whereas lower hematocrit levels lead to less viscous blood, producing larger spots. These variations can affect the uniform distribution of the drug across DBS paper [23]. To mitigate volume and hematocrit-related effects, a larger punch size was used to obtain DBS samples with a bigger diameter cut. In addition, blood spots were prepared with precise volume control by spotting 0.2 mL of blood onto a Whatman 903 demarcated circle [24]. This standardized approach effectively minimized the impact of hematocrit variation, ensuring consistency in DBS analysis.

Bioanalytical method validation (MV)
In November 2022, the USFDA published ICH M10 guidelines to provide standardized recommendations for bioanalytical MV and bio-study sample analysis. These guidelines were followed for the MV of Remdesivir using DBS technique [25]. The validation process included assessment of linearity, selectivity, matrix effect, autosampler carry over test (ASCOT), precision and accuracy batch (PAB), sensitivity, specificity, within and between run precision, and accuracy, and stabilities were evaluated during MV. Under stabilities, essential bench top (BT) stability and long-term (LT) stability were evaluated.

Selectivity and specificity and ASCOT
Selectivity was assessed using different human blood clots. One Blank sample from each lot; likewise, multiple blank samples were processed along with one set of freshly spiked, prepared CC standards, and batch evaluation QCs.

Specificity refers to capability of a bioanalytical method to identify and distinguish the analyte from other substances like medications likely to be used alongside intended treatment. To evaluate specificity, co-administered drugs were spiked to achieve Cmax values in injected samples as Caffeine (10 µg/mL) [26], Ibuprofen (15 µg/mL) [27], Diclofenac (100 µg/mL) [28], Paracetamol (10 µg/mL) [29], Ondansetron (40 ng/mL) [30], and Ranitidine (800 ng/mL) [31].

ASCOT was performed by injecting DBS processed samples in the sequence as extracted blank, extracted upper limit of quantification (ULOQ), extracted blank, extracted Lowest limit of quantification (LLOQ), extracted blank, extracted ULOQ, extracted blank, and at last extracted LLOQ blood samples.

The responses of interfering peaks at retention time (RT) of the Remdesivir in blank samples of selectivity, specificity and Blank (after 1st and 2nd injection of extracted high sample) in ASCOT sample should be <20% of response of LLOQ sample at 50 ng/mL and responses of interfering peak at RT and m/z of Remdesivir D5 shall be <5% of mean response of Remdesivir D5 observed in CC and QC samples.

Matrix effect

Matrix effects were evaluated by preparing low- and high-QC (150 and 4000 ng/mL) samples. Remdesivir was spiked into different lots of human blank blood containing K_2EDTA as an anticoagulant to generate these controls. These samples were processed using the DBS technique and analyzed alongside freshly prepared CC standards and batch qualifying QC samples. For all matrix lots, QC samples accuracy was required to fall within 85–115% and the coefficient of variation to be maintained at ±15%.

Recovery

Recovery was determined by comparing Remdesivir response at lower quality control (LQC), middle quality control (MQC), and high quality control (HQC) with detector response obtained from recovery samples with extracted spiked QC samples. Precision for mean % recovery obtained from each QC levels shall be ≤15% for both Remdesivir and Remdesivir D5.

Sensitivity and PAB

To determine sensitivity, six individual aliquots of LLOQ samples, along with one set of CC standards and batch qualifying QC samples, were processed and analyzed on different occasions using DBS. The signal-to-noise (S/N) ratio for all LLOQ samples should be ≥10.

PAB batches consisting of Blank, zero, 1 set of CCs, and 5 set of QCs at LLOQ QC, LQC, MQC, and HQC level processed using an optimized extraction method with DBS technique in human blood. Acceptance criteria of blank were same as selectivity blank samples. Calibration standards back-calculated concentration (BCC) must be within ±15% of nominal concentrations, whereas for LLOQ, it can be within ±20%. Out of all analyzed calibration standards, 75% must meet the above criteria.

Stabilities

For DBS, BT and LT stability are critical parameters. For each stability assessment, five separate aliquots of LQC and HQC samples (stability samples) were spotted onto DBS paper. The BT stability samples were stored at room temperature on the bench for 7 h, whereas LT stability samples were kept in a desiccator at room temperature for 16 days. After stability duration, stability samples along with one set of freshly spiked CC standards and freshly spiked batch qualifying QC's were processed and analyzed. The mean back-calculated values of the stability samples were compared to their nominal values, and percent change was required to be no more than 15%.

RESULTS AND DISCUSSION

Method development

Optimization of mass spectrometric conditions

During the tuning process of mass spectrometer, mass parameters were adjusted initially for Remdesivir in the electrospray ionization source under MRM mode to achieve optimal responses. Afterward, Remdesivir D5 was tuned to get an optimized response.

The obtained mass parameters are presented in Table 2.

In positive ion mode consistent, strong and reliable response was observed.

Optimization of the chromatographic condition

During initial trials, split peak was observed with the Hypersil Gold C18 column (150 × 4.6 mm, 5 µm). After trying multiple columns, finally optimal peak shape was achieved with Waters Symmetry C18 column (300Å, 5 µm, 3.9 mm × 150 mm). The injection volume was optimized at 20 µL, with a flow rate of 0.7 mL/min, an autosampler temperature set at 10°C, and a column oven temperature of 40°C. The carryover issue was later resolved with Acetonitrile: 10 mM Ammonium Acetate [90:10% v/v] needle rinsing solution.

Optimization of the DBS technique along with extraction method

Out of multiple trials, Whatman 903 card consistently showed uniform spreading of spiked blood within the designated circle. For soaking spotted disc, a 1% acidified methanol solution with formic acid yielded good recovery after shaking and vibration using Vibramax instrument at 2000 rpm. Further improvements in extraction efficiency were achieved by acidifying the extraction solution to facilitate Remdesivir release. An additional cleanup step involving evaporation and reconstitution with the mobile phase was optimized for better sensitivity. With this optimized DBS procedure, a Gaussian peak with acceptable recovery was obtained, which establishing this extraction method as optimized for MV using the DBS technique.

MV

Selectivity, specificity and ASCOT

Compared to LLOQ (50 ng/mL) of Remdesivir during the selectivity assessment, no interference was observed at the RT of Remdesivir and Remdesivir-D5 in blank blood. Representative blank blood chromatograms from one human blood lot shown in Fig. 3.

In addition, no interference was detected at RT and m/z of Remdesivir and Remdesivir-D5 in specificity samples. Based on these results, the DBS method was found to be selective across multiple blood lots and specific for human blood, even in the presence of concomitant drugs.

In ASCOT assessment, following the first and second injections of extracted ULOQ samples, responses of the extracted blank samples

Table 2: Mass parameter in MRM mode for Remdesivir and Remdesivir D5

Compound dependent parameter							
Compound Name	**Q1**	**Q3**	**Q1 resolution**	**Q3 resolution**	**Source fragmentation**	**Collision energy (eV)**	**Chromatographic peak width**
Remdesivir	603.3	327.8	0.7	1.2	0.0	5.0	10
Remdesivir D5	608.0	332.9	0.7	1.2	0.0	20.0	10
Source dependent parameter							
Dwell (ms)	**CID gas**	**Ion spray voltage (V)**		**Vaporizer temperature (°C)**		**Sheath gas**	**Aux gas**
331.1	1.5	4000.0		350.0		45.0	1.0

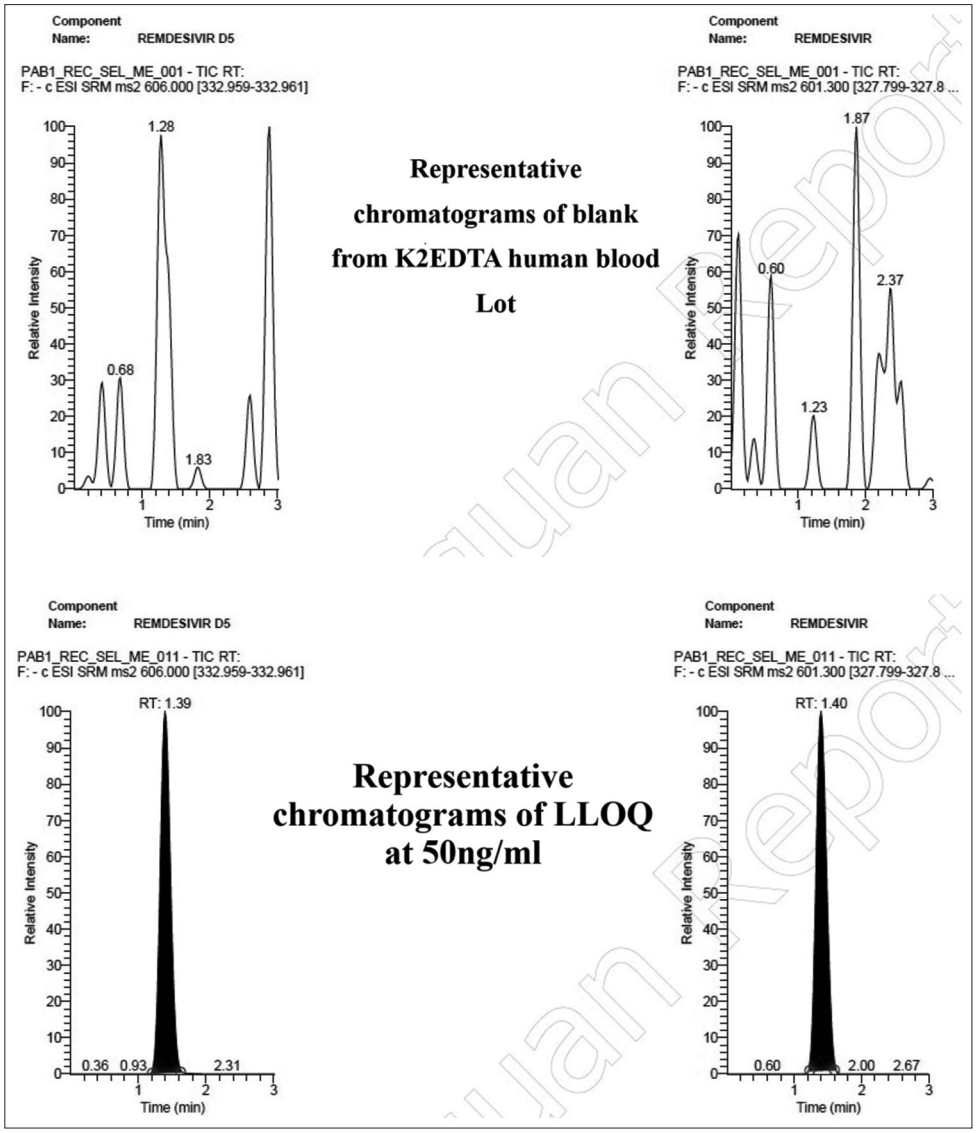

Fig. 3: Representative chromatograms from the selectivity experiment

show zero interference, which was within the acceptance criteria. Hence, no autosampler carry over observed for remdesivir and Remdesivir D5 with human blood bioanalytical method, so method and system are considered as free from carryover of Remdesivir and Remdesivir D5.

Matrix effect

Precision for both LQC and HQC samples observed as 1.70 and 1.91, respectively, which was within the acceptance criteria (≤15%). Moreover, the accuracy of these QC samples ranged between 85% and 115% of their nominal values, which meets the acceptance criteria for evaluating matrix effects.

Consequently, the bioanalytical method for human blood was determined to be free from significant matrix effects.

Recovery

Batch qualifying QC samples met acceptance criteria, with precision for mean % recovery at each QC level being ≤15% for both Remdesivir and Remdesivir D5. The recovery was determined 98.93% for remdesivir and 96.17% for remdesivir D5.

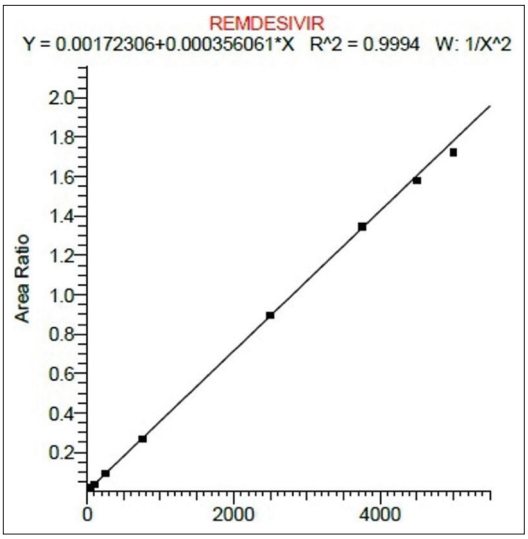

Fig. 4: Representative regression analysis of a calibration curve for human blood

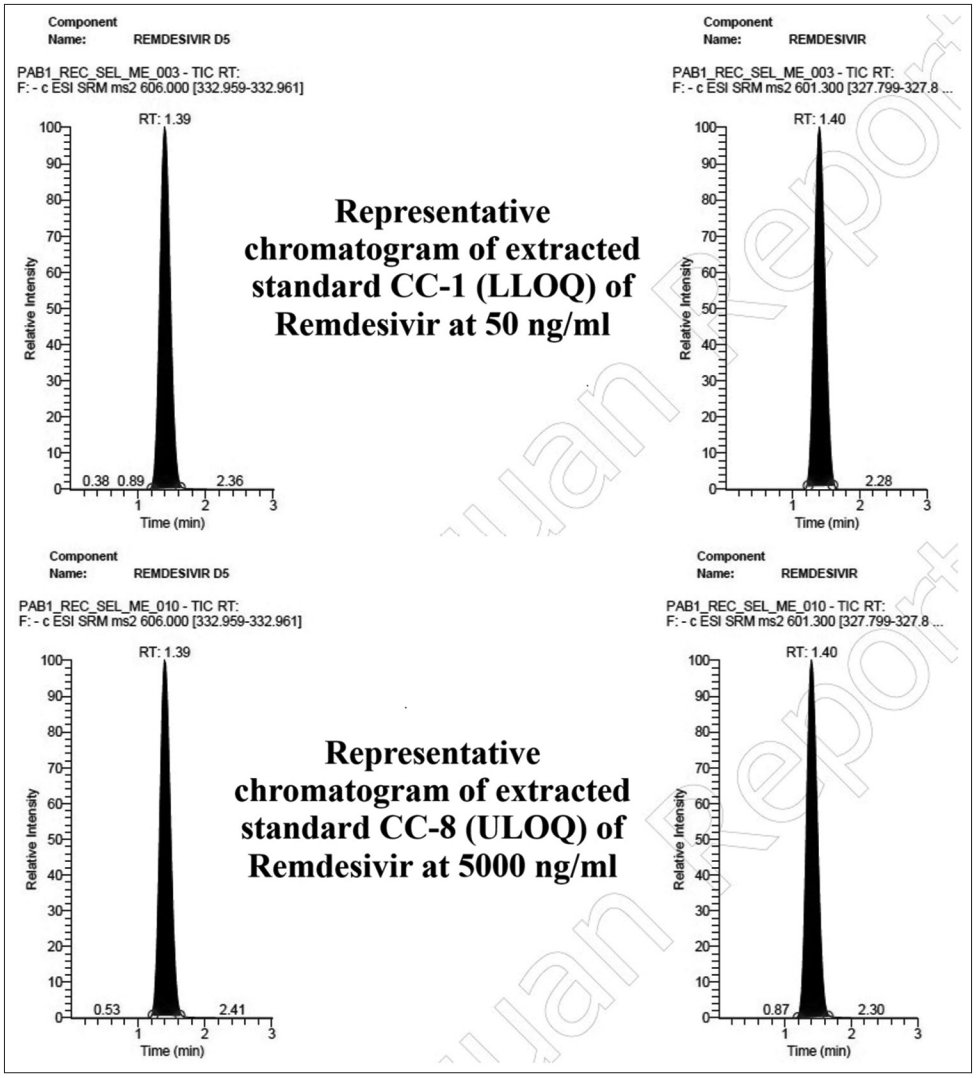

Fig. 5: Representative chromatogram of extracted standard calibration curve (CC)-1 and CC-8 from precision and accuracy batch

Table 3: CC data for precision and accuracy batches

CC ID	Nominal concentration (ng/mL)	Mean (ng/mL)	CV (%)	Mean % nominal
CC1	50.0	48.9±0.23	0.48	97.93
CC2	100.0	103.5±1.97	1.91	103.58
CC3	250.0	254.1±6.67	2.63	101.68
CC4	750.0	744.9±1.37	0.18	99.32
CC5	2500.0	2550.5±48.62	1.91	102.02
CC6	3750.0	3744.1±45.07	1.20	99.85
CC7	4500.0	4441.0±12.66	0.29	98.69
CC8	5000.0	4847.0±16.14	0.33	96.94

All values are expressed as mean±SD, n=2

In the developed DBS method for human blood, good and consistent recovery was observed for both remdesivir and remdesivir D5.

Sensitivity and precision accuracy batch

A signal-to-noise ratio exceeding 140 was observed for all LLOQ samples across various batches, which was well above the acceptance limit of 10. Based on these results, DBS-assisted human blood bioanalytical method was determined to be sensitive at 50 ng/mL.

In all PA batches, both blank and zero samples were free from any interference at the Remdesivir's RT. Moreover, BCCs of non-zero calibration standards as shown in Table 3 did not exceed ±15% of their nominal concentrations and these standards met criteria with

Table 4: Within and between run precision and accuracy

QC level	Within-run			Between run		
	Mean BCC (ng/mL)	%CV	% Nominal	Mean BCC (ng/mL) n=10	%CV	% Nominal
LLOQ QC (50.0 ng/mL)	49.1±1.35	2.77	98.23	48.0±1.60	3.33	96.04
LQC (150.0 ng/mL)	149.0±2.01	1.35	99.37	149.2±3.05	2.05	99.49
MQC (2500.0 ng/mL)	2696.6±74.82	2.77	107.87	2709.4±70.17	2.59	108.38
HQC (4000.0 ng/mL)	4306.4±116.65	2.71	107.66	4315.0±81.62	1.89	107.88

All values are expressed as mean±SD, n=5

Table 5: Bench top and long-term stability assessment in blood

Parameters	Bench top stability after 7 h		Long-term stability after 16 days	
	LQC	HQC	LQC	HQC
Nominal concentration (ng/mL)	150.0	4000.0	150.0	4000.0
Mean BCC	151.2±2.13	4434.5±123.26	148.9±2.26	4437.2±93.77
%CV	1.41	2.78	1.52	2.11
% Change	−0.83	−10.86	0.67	−10.93

All values are expressed as mean±SD, n=5

a determination coefficients (r2) value of 0.9978, as shown in Fig. 4. As represented in Table 4, within run and between run precision for QCs ranged from 1.35% to 3.33% whereas within run and between-run accuracy ranged from 96.04% to 108.38%. Representative chromatograms for CC1 (LLOQ) and CC8 (ULOQ) are shown in Fig. 5.

In completed batch, 67% of overall QCs with 50% at each level meets acceptance criteria.

Stabilities

Percent change of LQC and HQC samples were within the acceptance criteria of ±15%, while comparing the mean of back-calculated values of stability samples against nominal values after BT stability of 7 h and LT storage of 16 days (Table 5).

From stability evaluation confirmed that remdesivir stable on the bench at room temperature for 7 h as well as stable in a desiccator for 16 days, which will be sufficient to complete bioanalysis after sample collection in TDM or clinical studies.

CONCLUSIONS

The developed DBS method, although involving complex processing steps, is both green and environmentally friendly. It is highly suitable for TDM and clinical studies, particularly due to its low blood volume requirement – a critical advantage during virulent outbreaks. Bioanalytical studies using blood from multiple human donors have confirmed that the DBS technique does not lead to overestimation of Remdesivir. The LC-MS/MS bioanalytical method, employing Remdesivir D5 as an IS in human blood containing K_2EDTA, was successfully validated. This validation covered key parameters including system suitability, carryover, selectivity, ruggedness, sensitivity, specificity, matrix effect, recovery, and essential stability studies, with a calibration range of 50–5000 ng/mL. Despite advanced instrumentation and complex processing steps, all MV parameters met predefined criteria in accordance with ICH M10 guidelines, thereby confirming both the accuracy and precision of the developed method for remdesivir detection.

AUTHOR CONTRIBUTIONS

All authors are contributed equally.

DECLARATION OF COMPETING INTEREST

The authors completed this research work without any influence or financial interests or personal relationships, and the same reported in this paper.

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