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A Comparative Study of Human Dried Blood Spot and Conventional Plasma-Based Bioanalytical Methods Using Lc-Ms/Ms.

Dr Subhranshu Panda[1], Tushar Chavan[2], Dr Ravindra Bhavsar[3]

[1]Professor and Director, Department of Analytical, School of Pharmaceutical Sciences, Jaipur National University, Jagatpura, Jaipur, Rajasthan, 302017, India

[2]Research Scholar, Department of Analytical, School of Pharmaceutical Sciences, Jaipur National University, Jagatpura, Jaipur, Rajasthan, 302017, India

[3]Department of Clinical, Pharmadesk Solutions Pvt Ltd, Navi Mumbai, Maharashtra 400710, India

Corresponding Author:

Tushar Chavan, Research Scholar, Department of Analytical, School of Pharmaceutical Sciences, Jaipur National University, Jagatpura, Jaipur, Rajasthan, 302017, India

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KEYWORDS

Remdesivir, Dried Blood Spot; Bioanalytical Method Validation; Bioanalysis; Clinical study; ICHM10; LC-MS/MS

ABSTRACT:

Introduction: The conventional blood collection and analysis process has been made quicker by the use of DBS technology in the fields of therapeutics and health sciences. For pharmacokinetic and toxicokinetic investigations, DBS is expected to be a viable substitute for well-established liquid bio methods and potentially even outperform them provided if it comparable to conventional human plasma bioanalytical method.

Objectives: The purpose of this comparison study was to compare validated dried blood spot (DBS) method with traditional gold standard human plasma method for determining Remdesivir using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The comparison should be using both statistical and graphical approaches to nullify bias.

Methods: An electrospray ionization source with multiple reaction monitoring was used on a Thermo Fisher Scientific Accela HPLC system with a TSQ ENDURA mass spectrometer. DBS extraction method involve use of Whatman 903 paper whereas human plasma method involves protein precipitation. Statistical analyses for method comparison included Bland-Altman and paired t-tests. Box-and-whisker plots and Scatter plots with Deming regression were used in the graphical evaluations.

Results: Both methods were validated according to the ICH M10 guidelines. The graphical and statistical comparison revealed that the DBS approach works within acceptable limits when compared to the human plasma method.

Conclusions: Both methods were effectively examined and compared throughout a linearity range of 50-5000 ng/mL. The validated DBS method for Remdesivir is a strong and dependable alternative to traditional plasma-based sampling. Because of its minimal invasive nature, the DBS technique is ideal for clinical and therapeutic drug monitoring applications.

1. Introduction

Conventional human plasma bioanalysis remains the predominant technique in pharmaceutical research, widely adopted across the industry. Its extensive application is attributed to broad acceptance by both researchers and regulatory authorities, particularly in studies submitted for regulatory review. This approach offers several advantages for bioanalytical scientists, including the availability of large sample volumes, which facilitates the quantification of low-concentration analytes and allows for multiple analyses from a single sample. However, these benefits come at a cost[1]. Plasma-based methods are inherently more invasive due to significant blood volume requirements, often necessitating multiple venipunctures from clinical trial

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participants[2]. Furthermore, these techniques involve complex intermediate steps such as plasma separation and require resources for separation and ultra-cold storage of biological samples until analysis[3]. These limitations have prompted the exploration of alternative sampling methods, such as DBS analysis, which offers safer and more convenient alternative to blood collection. In DBS technique, a drop of blood is applied to a specialized filter paper, where it dries and can be later processed for the extraction and quantification of target analytes[4]. This method provides numerous advantages, including minimal blood volume requirements, simplicity of collection, suitability for self- or unskilled sampling and reduced need for specialized storage conditions [2,5]. These features have made DBS particularly attractive in pediatric studies and have enabled its use in at-home sampling for monitoring patient compliance[6,7]. Despite of these advantages, there is still a lack of sufficient published literature validating DBS as a full replacement for conventional plasma or whole blood bioanalysis in regulatory contexts.

In this study, we aimed to develop and validate a sensitive bioanalytical method for quantifying Remdesivir in both human plasma and DBS matrices. Each method was validated over a linearity range of 50–5000 ng/mL, based on the reported Cmax of Remdesivir (2229 ng/mL). Comparative evaluation between the two methods was performed using both statistical and graphical analyses to assess equivalency. Bioanalysis was carried out using a Thermo TSQ ENDURA triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in multiple reaction monitoring (MRM) mode. Method validation followed the guidelines outlined in the ICH M10 regulatory framework for bioanalytical method validation[8].

Volumetric Absorptive Microsampling (VAM) is different analytical technique and 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. So, it cannot consider alternative to developed DBS method in this research[9]. 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[10]. In contrast, our DBS method utilizes whole blood directly, thus significantly reducing sample processing complexity and blood volume requirements. Importantly, to our knowledge, no prior studies have conducted a direct comparison of DBS method accuracy against the gold-standard plasma LC-MS/MS method for Remdesivir. Our study addresses this gap by validating the DBS method according to international guidelines and demonstrating its comparability with the plasma-based assay through correlation analysis. These findings support the interchangeability of the DBS method with conventional plasma bioanalysis for Remdesivir.

2. Material and Method

Material, chemicals and reagents

Merck supplied Methanol, Acetonitrile (LC-MS grade), Ammonium Acetate, Ammonium Formate (HPLC grade) and Formic Acid (Emparta grade) was used. Deionized water (LC-MS grade) was created in-house using a Millipore water purification system. Whatman 903 DBS cards with 17mm mean spot diameter and 9 sec/100µl mean blood absorption time were bought from Cytiva, India. Vivan Life Sciences India provided Remdesivir (Purity 99.74 %) and Remdesivir D5 (99.8 % D atom isotopic enrichment). 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%) required for specificity obtained from same manufacturer.

Human Plasma and blood

After ethics committee approval for the collection of whole blood from human volunteers, a phlebotomist at the Clinical Lab, Navi Mumbai, India, took blood from consenting donor who had not taken any of the research medicines previous to donation. Blood for bioanalytical techniques was collected into tubes with dipotassium ethylenediaminetetraacetic acid (K_2EDTA) anticoagulant tubes and kept at 4°C overnight before bioanalytical activities whereas separated plasma stored at -20°C.

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Preparation of stock and working solutions

Separate Remdesivir's calibration curve (CC) and quality control (QC) analyte stock solutions were prepared in Methanol with concentrations of 1 mg/mL, whereas Remdesivir D5 stock also prepared in Methanol. The stock solutions were initially utilized to prepare CC and QC required till initial method validation whereas during evaluation of stabilities again separate stocks were prepared. CC have concentration ranges of 50-5000 ng/mL concentrations of Remdesivir. QCs were prepared at different concentrations as lower limit of quantification QC sample (LLOQ QC) at 50 ng/ml, low QC samples (LQC) at 150 ng/ml, medium QC sample (MQC) at 2500 ng/ml and high QC sample (HQC) at 4000 ng/ml. Common CC and QC dilutions were used for human plasm as well as DBS method development as well as for validation.

For Human plasma method, spiked CC and QC plasma samples were individually spiked in ria tubes. For DBS, 0.200ml aliquots of each CC and QC were spotted on Whatman 903 DBS cards. The samples were dried for 1 hr using air dryer and stored in sealed zip-lock bags with desiccant sachets at room temperature.

Human plasma extraction procedure

In extraction trials, liquid-liquid extraction and protein precipitation were explored initially, followed by liquid-liquid or solid phase extraction. Protein precipitation analyte peak showed a rather good response with little recovery. In the final trial, 0.200 ml of spiked human plasma was precipitated using acidified acetonitrile instead of methanol solvent. The sample was vortexed for 15 minutes, centrifuged for 10 minutes at 4000 rpm and the supernatant was injected over LC-MS/MS. A decent peak shape with satisfactory recovery and a symmetric peak were obtained. So, this extraction approach was considered as the optimal method for method validation.

DBS extraction procedure

Several DBS studies from diverse sources were examined throughout the creation of the approach. The DBS procedure was optimized using a hole punch plier (ticket punch) with a 10 mm diameter cut and a standardized blood spotting volume of 0.2 mL. Following drying, multiple extraction trials were carried out to effectively extract Remdesivir from DBS paper. In the optimized extraction method, a 10 mm DBS paper disc was vortexed and centrifuged in 1 mL of acidified methanol. The supernatant was evaporated at 40°C and 10 psi air pressure with a N_2 Fastvap Nitrogen Evaporator. Before LC-MS/MS analysis, the dried residue was reconstituted in an optimized mobile phase containing methanol and 10 mM ammonium acetate (pH 3) in a 95:5% (v/v) ratio. To reduce contamination from the hole punch, CC and QC samples were punched from low to high concentrations. To prevent carryover, the punch was rinsed between samples with 100% methanol and a blank paper punch.

Chromatographic and LC-MS/MS condition

During this research, Thermo Fisher Scientific Accela HPLC system, coupled with a TSQ ENDURA mass spectrometer, was used for analysis. Data acquisition and processing were performed using LCquan 3.0 software. Chromatography was performed on a Waters Symmetry C18 (300Å, 5 µm, 3.9 mm × 150 mm) column. Given 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. Finally, acidified acetonitrile:10 mM ammonium acetate mobile phase was selected as final condition. Optimised flow Rate was set to 0.7 ml/min with injection volume of 20µl.

Mass parameters were tuned on LC-MS/MS - TSQ ENDURA (Thermo Fisher Scientific) without integrating HPLC system. During tuning process, only mass profile was activated. A 100 ng/mL solution of Remdesivir in Methanol was prepared and continuously infused into ESI source using a syringe infusion pump with flow of 10 µL/min for direct mass spectrometric optimisation. Objective of tuning was to obtain optimised Quadrupole-1 (Q1) and Quadrupole-3 (Q3) responses by systematically adjusting compound and source-dependent parameters to achieve optimal ionization and detection.

Bioanalytical method validation

Human plasma and DBS analysis was completed in different analytical runs on separate occasions.

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Selectivity and specificity

Selectivity assessed using different human plasma lots for conventional and human blood lots for DBS method validation. 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 analyte from other substances like medications likely to be used alongside intended treatment. To evaluate specificity, co-administered drugs were spiked in plasma as well as spotted in DBS to achieve Cmax values in injected samples as Caffeine (15 µg/ml)[11], Ibuprofen (15 µg/ml)[12], Diclofenac (100 µg/mL)[13], Paracetamol (10 µg/mL)[14], Ondansetron (40 ng/mL)[15] and Ranitidine (800 ng/mL)[16].

Matrix effect

Matrix effects were assessed by processing LQC and HQC samples at concentration of 150 and 4000 ng/ml, respectively in human plasma as well as with DBS card. To construct the controls, these samples were processed into several lots of human blank plasma and blood. QC samples for all matrix lots needed to be accurate within 85-115% and have a coefficient of variation (CV) of ±15%.

Recovery

For Recovery determination in human plasma, processed LQC, MQC and HQC samples compared with by comparing Remdesivir response observed at blank processed samples with aqueous samples spiked directly into blank extract. Similarly, to evaluate recovery in DBS, instead of spiked plasma, spotted DBS was processed at LQC, MQC and HQC samples. Percent recovery obtained from each QC levels shall be ≤ 15 % for both Remdesivir and Remdesivir D5.

Sensitivity and precision accuracy batch

To evaluate sensitivity, six individual aliquots at the LLOQ, along with one complete set of CC standards and batch-qualifying QC samples were processed and analyzed on separate occasions using the conventional human plasma and DBS technique. Each LLOQ sample was required to exhibit a signal-to-noise (S/N) ratio of at least 10.

Precision and accuracy batch (PAB) were prepared using the optimized DBS extraction method in human blood. The acceptance criteria for blank samples were consistent with those established during selectivity assessments. The back-calculated concentrations of the CC were required to be within ±15% of their respective nominal concentrations, except for the LLOQ standard, which was permitted a deviation of up to ±20%. A minimum of 75% of the non-zero CC were required to meet these acceptance criteria.

Stabilities

For each stability test, five separate aliquots of LQC and HQC samples were spiked in human plasma and applied to DBS cards. For Bench Top (BT) stability evaluation, stability samples were stored on the laboratory bench under ambient circumstances. For Long Term (LT) stability evaluation, human plasma samples stored at -20°C and DBS stability samples were kept in a desiccator at room temperature. The mean back-calculated concentrations of the stability samples were then compared to their nominal concentrations, with the percent deviation required to remain within ±15% to be deemed acceptable.

Statistical Comparison

The Bland-Altman analysis is a common approach used in research to compare the agreement of two alternative techniques of measuring the same medical parameter. This method is used to determine the magnitude of differences in plasma concentrations with the DBS method by comparing the DBS and plasma measurement methods[17]. If bias is large or points trend upward/downward, systematic or proportional bias may exist. Similarly, if many points fall outside limit of agreement (LOA), then it will indicate methods may not be interchangeable

The Paired t-Test compares the means of two sets of observations. The observations must be given at random to each of the two groups so that any difference in reaction is related to the treatment rather than any other reason. This test can be used to observe the same sample before and after an event. To analyse the pair t-test, data QC samples from method validation were employed, with the Y axis showing back estimated concentrations and the X axis showing human plasma (HP) and DBS QCs. The Shapiro-Wilk test was used to determine the

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normal distribution of differences while computing the t-test. Bland-Altman analysis and Pair-t tests primarily provide mean difference (Plasma - DBS), t-statistic (t-value), degrees of freedom (df = n-1) and p-value, which is a two-tailed probability. These tests were carried out using MedCalc® version 23.1.7 software.

Graphical Analysis

Box whisker plot mainly used for visualizing data distribution and variability. Data distribution analysis of the results was done to determine median ratios and outliers using Box and Whisker Plots.

A scatter plot visualizes the relationship between two variables. When both variables contain measurement errors in a bioanalytical method comparison, a Deming regression line is employed instead of ordinary least squares (OLS) regression. Although human plasma is the gold standard approach, it also accounts for standard error; therefore, Deming regression allows for measurement errors in both the X and Y variables.

3. Results and Discussion

Chromatographic and LC-MS/MS condition

The ESI source in positive ion mode responded substantially stronger during tuning than the negative ion mode. In positive polarity, the product ions of Remdesivir and Remdesivir-D5 showed strong peaks at Q1 with mass-to-charge ratio (m/z) values of 603.3 and 608.0, respectively. When Q1 was fixed, a scan of Q2 yielded product ions at m/z 327.8 for Remdesivir and 332.9 for Remdesivir-D5, as shown in Fig.1. Mass spectrometric parameters were optimized using collision energies of 5.0 and 20.0 eV, a chromatographic peak width of 10, a dwell time of 331.1 ms, a CID gas pressure of 1.5 psi, an ion spray voltage of 4000 V, a vaporizer temperature of 350°C, a sheath gas pressure of 45.0 psi and an auxiliary gas pressure of 1 psi.

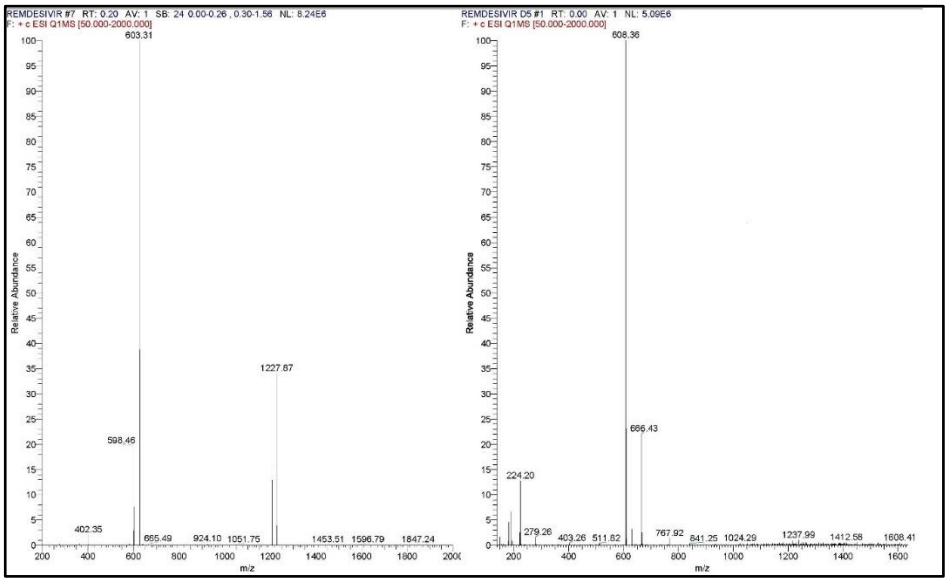

Fig. 1: Parent ion mass spectra of Remdesivir and Remdesivir D5

Following mass tuning, multiple trials were conducted using different HPLC columns and varying mobile phase compositions to optimize chromatographic conditions. Column oven temperature of 40°C with autosampler Temp 10 °C helps to achieve run time of 3 minutes which is shortest of all available methods. The injection needles were cleaned with an Acetonitrile: 10mM Ammonium Acetate [90:10% v/v] and the autosamplers were kept at around 10°C. The objective was to achieve a well-defined peak shape without fronting or tailing, along with a satisfactory chromatographic response.

Method validation

Selectivity and specificity

During the selectivity test, no interference was detected at the retention times (RT) of Remdesivir and Remdesivir-D5 in blank blood and human plasma lots

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when compared to Remdesivir's LLOQ (50 ng/mL). Similarly, no interference was seen at the RT and m/z values of Remdesivir and Remdesivir-D5 in specificity samples from human plasma or DBS. Representative chromatograms from DBS method shown in Fig.2.

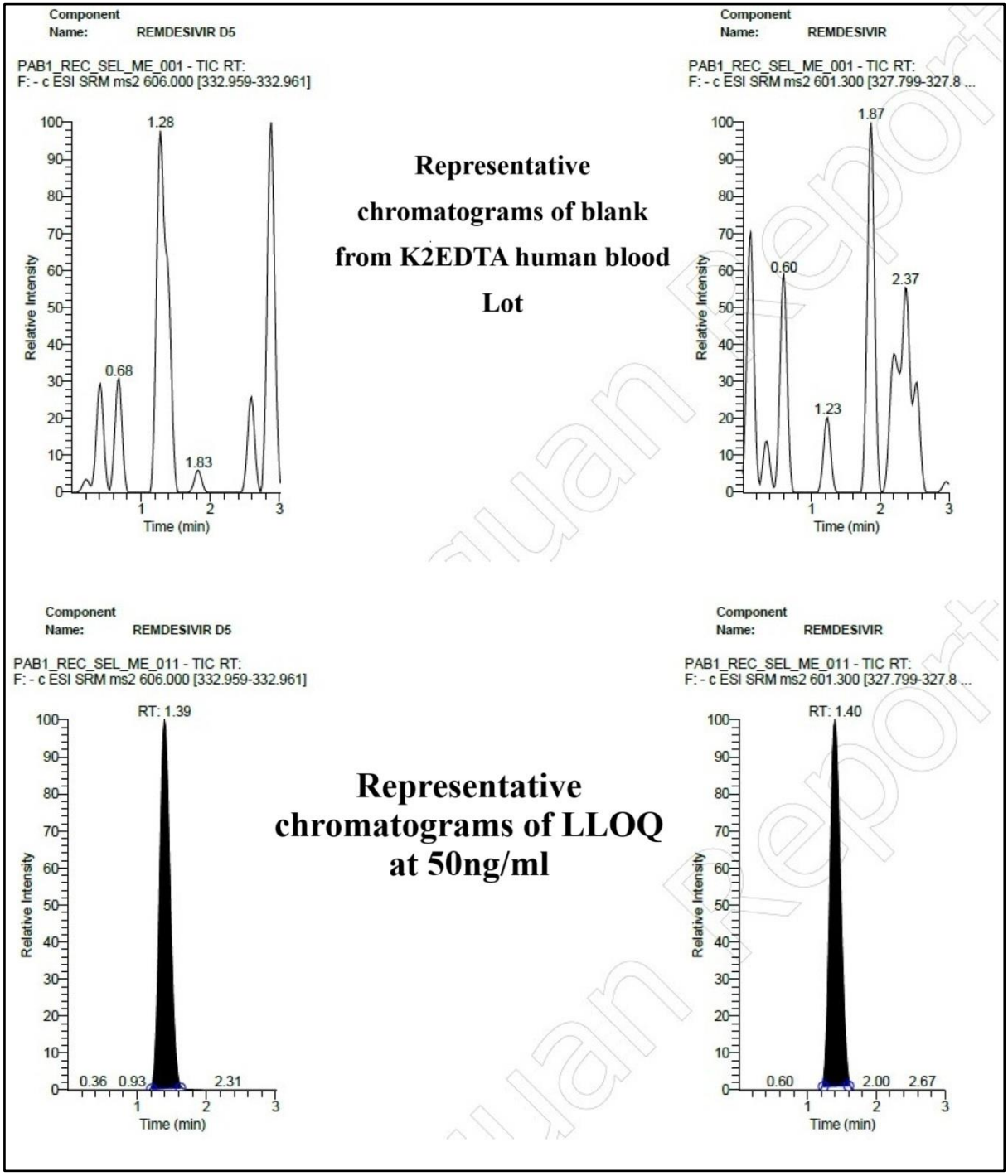

Fig. 2 Representative chromatograms from selectivity experiment

These data validate the new method's selectivity across various plasma and blood lots. Furthermore, the approach proved specificity for human plasma and blood, even in the presence of other drugs.

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Matrix effect

The precision for LQC and HQC samples was 2.32% and 1.68% in plasma and 1.70% and 1.91% in DBS, all within the acceptable limit of ≤15%. Both matrices had an accuracy range of 85% to 115% of nominal values, meeting the matrix effect evaluation requirements. Thus, the approach was shown to be free of significant matrix effects in both human plasma and blood.

Recovery

Remdesivir and Remdesivir-D5 had a mean % recovery precision of ≤15% at all QC levels for both human plasma and DBS samples. Remdesivir's mean recovery was 91.87% in plasma and 98.93% in DBS samples. The established procedures for both matrices produced consistent and reliable recovery of Remdesivir and its internal standard, Remdesivir-D5.

Sensitivity and precision accuracy batch

The signal-to-noise ratios for all LLOQ samples were greater than 179 for human plasma and 140 for DBS, much exceeding the acceptance criterion of 10, validating the sensitivity of both bioanalytical techniques at 50 ng/ml.

Blank and zero samples from all PA batches exhibited no interference during Remdesivir's RT. Back-calculated concentrations for non-zero CC remained within ±15% of nominal values, with determination coefficients (r^2) of 0.9978 and 0.9994 for plasma and DBS, respectively. Within- and between-run precision for QCs was from 0.58-4.97% and 1.35-3.33%, respectively, while accuracy ranged from 95.22-110.74% and 96.04-108.38% for plasma and DBS. Furthermore, 67% of total QCs and 50% of QCs per level were within 85-115% of their nominal values in both matrices.

Stabilities

The percentage changes for LQC and HQC samples were within the acceptable limits of ±15% when comparing the mean back-calculated values of stability samples to nominal values. This was seen following BT stability at 4 hours for plasma and 7 hours for DBS, as well as LT storage for 16 days at -20°C in human plasma and at room temperature in DBS.

Statistical Comparison

Bland-Altman and Paired t-Test assessment was done after using MedCalc® version 23.1.7 software. Both analyses involve use of QC data from complete method validation as shown in Fig.3.

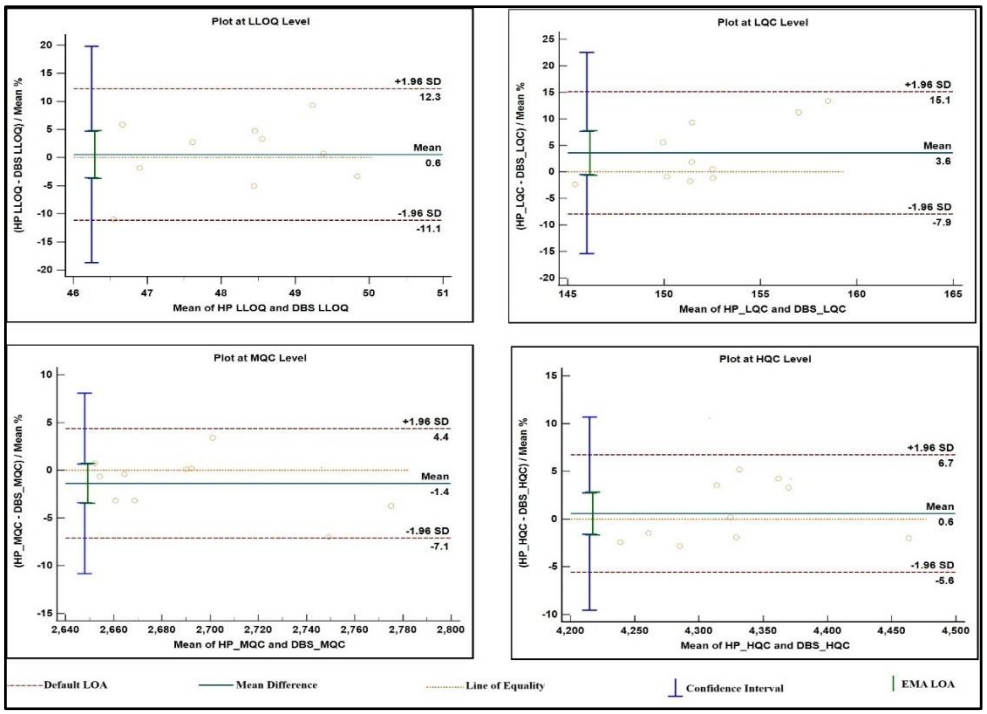

Fig. 3: Bland-Altman analysis plot for Human plasma and DBS method

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The majority of the points in all of these graphs are within the LOA, indicating that these two approaches have strong agreement at all QC levels and are interchangeable. The Bland-Altman plots show the arithmetic mean difference as a percentage, the limits of agreement (LoA) at ± 1.96 x SD (standard deviation) away from the mean differences, the EMA LoA (± 20% of the mean differences) and the 95% confidence interval (CI) for estimated precision.

As represented in table 1 for Paired t-Test evaluation, P-values calculated by the paired sample t-test were > 0.05 for all QCs at all four level (0.0861–0.7590), indicating no statistically significant differences between the DBS and human plasma QCs in term of concentrations as shown in Fig.4.

Table 1: Paired samples t-test evaluation after comparing human plasma and DBS bioanalysis

	Paired samples t-test			
QC Level	**LLOQ**	**LQC**	**MQC**	**HQC**
Mean difference between HP and DBS	-0.2852	-5.5753	37.3028	-25.3939
Standard deviation of differences	2.8517	9.1504	79.9496	135.7767
Standard error of mean difference	0.9018	2.8936	25.2823	42.9364
95% CI of difference	-2.3252 to 1.7548	-12.1211 to 0.9705	-19.8897 to 94.4953	-122.5227 to 71.7349
Test statistic t	-0.316	-1.927	1.475	-0.591
Degrees of Freedom	9	9	9	9
Two-tailed probability	P = 0.7590	P = 0.0861	P = 0.1742	P = 0.5688

HP: Human plasma method; DBS: Dried blood spot method; CI: Confidence interval

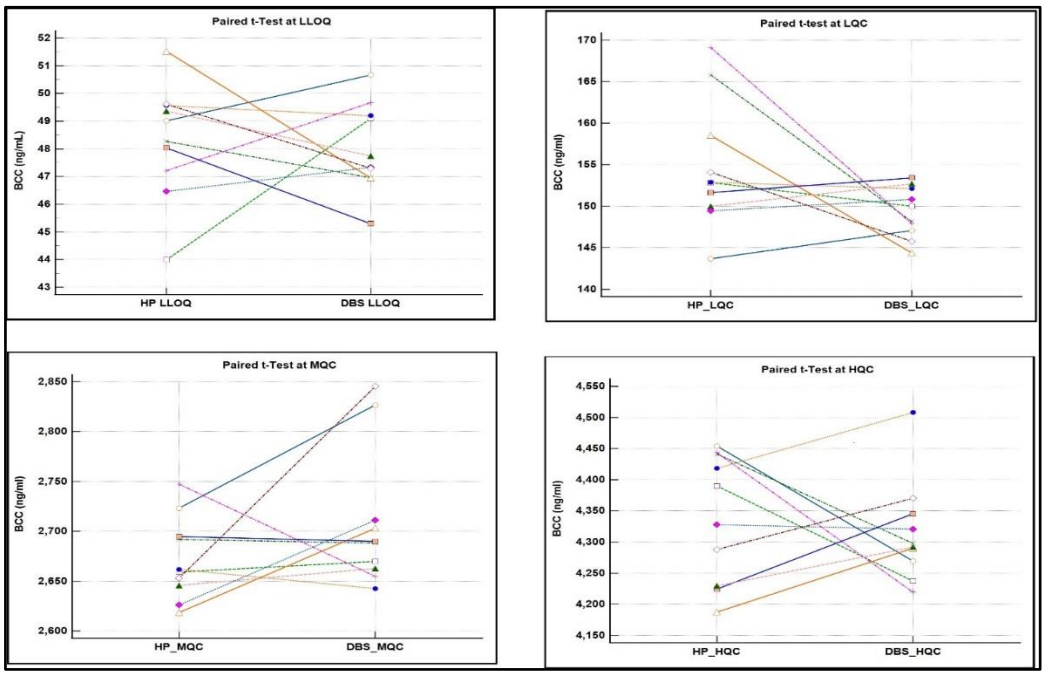

Fig. 4: Paired t-test to determine statistical significance differences between Human plasma and DBS method

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Graphical Analysis

Box and Whisker plots showed in Fig.5 indicate that the [DBS]/[Human Plasma] ratio is approximately normally distributed at all QC concentrations in human plasma as well as DBS method. From graph it can be easily understand that these ratios are in between 0.95 to 1.1 which are in very close agreement with each other.

All QC concentrations observed in Fig.6 are on regression line with no outliers detection. Deeming regression shows slope (m) as 1.001 and correlation coefficient (r) value as 1 indicates strong agreement between human plasma and DBS method.

All graphical methods show DBS as well as human plasma method as interchangeable and without any significant difference.

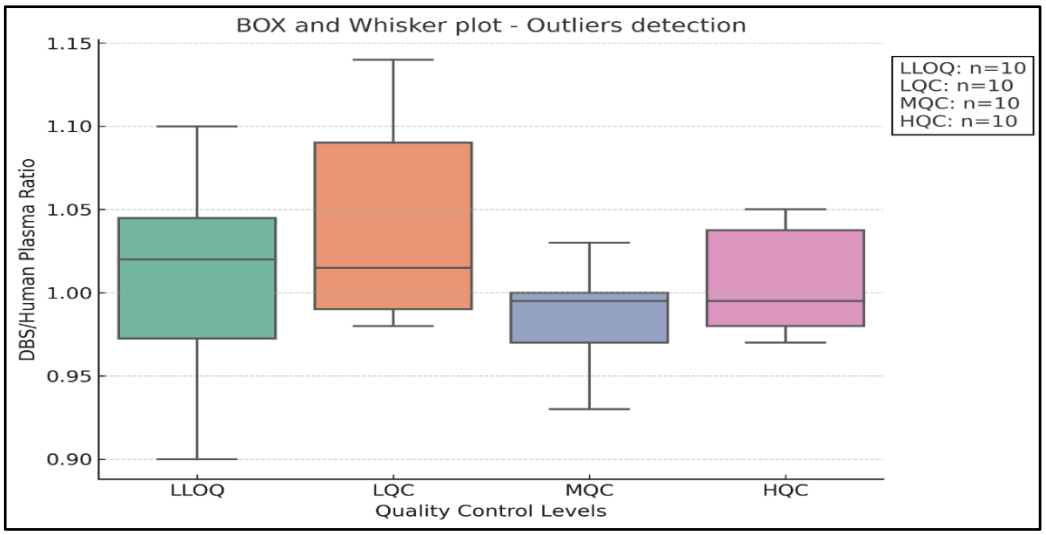

Fig. 5: Box and whisker plots of the ratios of DBS to plasma concentrations at all QC level

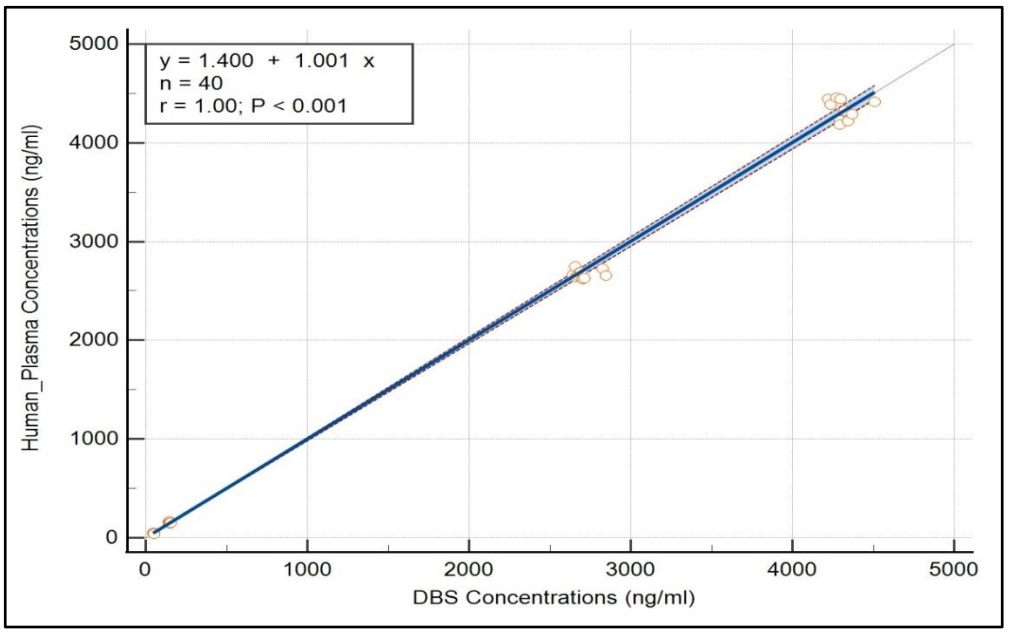

Fig. 6: Scattered plot with comparison of human plasma to DBS concentrations at all QC level

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4. Conclusions

We successfully developed and validated a reliable bioanalytical method for measuring Remdesivir in human plasma and dried blood spots. The DBS approach integrates novel processing processes while being green and environmentally sustainable. Despite the use of modern technology and difficult sample preparation processes, all validation parameters fulfilled the established criteria outlined in the ICH M10 recommendations, proving the method's capacity to accurately and precisely quantify Remdesivir. The DBS method's clinical application was further validated by statistical and graphical comparisons to the traditional plasma-based gold standard, revealing its potential utility in pharmacokinetic investigations, therapeutic drug monitoring (TDM) and adherence assessment. Future research should focus on expanding the use of the DBS approach in TDM, particularly in distant or resource-constrained situations where its inherent benefits such as minimum invasiveness and ease of sample transport are especially advantageous.

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