ABSTRACT

Objective: The current research work focus to simple and rugged bioanalytical method development and validation of brivudine in human plasma using high-performance liquid chromatography.

Methods: The analyte (Brivudine) and internal standard (Sofosbuvir) were extracted using the Solid Phase Extraction (SPE) technique. The chromatographic separation was accomplished by using Zorbax eclipse XDB-C$_{18}$ Column (150×4.6 mm, 5 μm) with a mobile phase consisted of Methanol: 0.5% Ortho-phosphoric acid (65:35%, v/v) respectively, at a flow rate of 0.7 mL/min. The developed method was validated by performing system suitability, carryover effect, linearity, selectivity, sensitivity, precision, accuracy, recovery, ruggedness, and stability studies. The method was validated as per USFDA guidelines.

Results: The selected chromatographic condition was found to efficiently separated brivudine (RT-3.55 min) and ISTD (RT-7.87 min). The assay demonstrated a linear dynamic range of 85.205 to 4500.246 ng/ml for brivudine in human plasma with r$^2$=0.99. Demonstrated the lowest limit of detection at 85.205 ng/ml. This method established an intra-run and inter-run precision within the range of 2.99-6.31%CV and 3.67-5.80%CV, respectively. Additional intra-run and inter-run accuracy were within the range of 97.55-105.37% and 99.27-102.15%, respectively. The mean percentage recovery of brivudine and ISTD studies proved good extraction efficiency and the robustness was also evaluated.

Conclusion: A simple, accurate, precise, linear and rugged RP-HPLC method was developed and validated for the estimation of brivudine in human plasma with K$_{E}$DTA anticoagulant and suitable for conducting BA/BE and TDM.

Keywords: Brivudine, Human Plasma, Liquid Chromatography, Solid Phase Extraction

INTRODUCTION

It is available as a 125 mg tablet and as a 0.1% ointment. Brivudine is a strong antiviral agent against the varicella-zoster virus and it is an analog of thymidine. It blocks viral replication by interfering with viral DNA through inhibition of deoxynucleotidase. It is almost completely absorbed in the gastrointestinal tract after oral administration, but due to extensive first-pass metabolism, bioavailability is about 30% and extensively bound to plasma proteins (95% binding), but is not displaced by concomitant drugs, including analgesics, cardiac glycosides, cytostatics and viostats and excreted mainly in the urine (65%) with less than 1% of unchanged brivudine found in urine, a minor fraction has been found in the faeces (21%). The terminal half-life has been established at 16 h [1].

It is also used as eye drops for the treatment of HSV-1 epithelial keratitis [3]. Oral brivudine is now approved in several EU countries (Germany, Italy, Austria, Belgium, Luxembourg, Spain, Portugal and Greece) for the treatment of herpes zoster in immune-compotent patients with the dose regimen of one tablet (125 mg) per day for 7 d [4].

This is expected to work by blocking the activity of a protein called 'heat shock protein 27' (Hsp27), which is found in high amounts in pancreatic cancer cells. Hsp27 is known to play a key role in 'chemoresistance'. When brivudine is given together with other anticancer medicines, it is expected to prevent the cancers cells from developing resistance to the medicines, helping to treat the disease [5].

Systematic (IUPAC) name: 5-[(1E)-2-bromoethenyl]-1-[2R, 4S, 5R]-4-hydroxy-5-hydroxymethyl]-oxolan-2-yl]-1,2,3,4-tetrahydropyrimidine-2,4-dione (fig. 1). It is freely soluble in Methanol and chemical formula is C$_{13}$H$_{15}$BrN$_{3}$O$_{5}$ with the molecular weight 335.135 g/mol [6].

Drug literature review reveals that few analytical quantification methods have been reported for the brivudine in bulk formulations, and biological matrices. Which includes UV spectrophotometric [8], capillary electrophoresis [9,10], high performance liquid chromatography coupled with ultraviolet (UV) detection [11,12] and ultra-high performance liquid chromatography-tandem mass spectrometric detection (UPLC-MS/MS) [13]. The present work designed to develop a simple, rugged, economic and validated RP-HPLC method for the determination of brivudine in human plasma with anti-coagulant K$_{E}$DTA.

MATERIALS AND METHODS

Chemicals and reagents

Brivudine and sofosbuvir bulk drugs were kindly provided as a gift sample by Sequent Labs Pvt. Ltd., Mangalore, India. Analytical grade of ammonium acetate purchased from Rankem Ltd., India and HPLC grade of methanol and acetonitrile purchased from Merck
Specialties Private Limited, India. Analytical grade orthophosphoric acid purchased from Rankem Ltd., India, Matrix: Human plasma (K$_2$EDTA-Anticoagulant) acquired from a registered blood bank.

**Instrument**

Quantitative HPLC was performed on a Shimadzu HPLC system with PDA detector and LC-MS solution software. An agilent Zorbax Eclipse XDB-C$_18$ column of 250 mm X 4.6 mm: i.d and 5µm particle size with 300° A pore size was used. Micro Weighing Balance (MXS)-Mettler Toledo, an Ultrasonic bath sonicator, Deep Freezer (-20±5 °C)-Thermo Fisher Scientific, Refrigerator (2-8 °C) -Thermo Fisher Scientific and Vortex-Scientific and Vortex-Spinix, Heidolph top were used in the study.

**Chromatographic condition**

Chromatography separation was achieved on a reverse-phase zorbax eclipse XDB-C$_18$ column (250×4.6 mm, 5µm) with isocratic elution by methanol: 0.5% O-phosphoric acid (65:35%, v/v) as the mobile phase. Chromatographic peaks were resolved by the mobile phase with a flow rate of 0.7 ml/min. The total analysis run time of 10 min and the analytical column temperature ambient.

**Sample preparation**

Exactly 0.200 ml of plasma sample was aliquoted and transferred 5 mg of Brivudine into 5 ml volumetric flask. The solution is made up to the mark by using Methanol dihent to get the concentration of 1.0 mg/ml and stored at 2-8 °C.

**Working standard preparation**

The working solutions for the calibration standards containing brivudine were prepared by dilution of the stock solution with the dihent 60% methanol solution (v/v). Calibration standards in human plasma were prepared by diluting the corresponding working solutions with screened blank plasma samples at the following concentrations: 85.205, 170.411, 340.141, 583.232, 972.053, 1620.088, 2700.147 and 4500.246 ng/ml for brivudine. The working solutions for the quality control (QC) samples were prepared at the following concentrations: 85.211, 240.030, 1500.188, 3500.204 and 17501.022 ng/ml for brivudine. ISTD working standard solution at a concentration of 500 ng/ml was prepared by diluting the stock solution (1.0 mg/ml) with the dihent. All stock solutions, working solutions, calibration standards and QCs were immediately stored at 2-8 °C.

**System suitability**

System suitability experiment was performed by injecting six repeated injections using a standard aqueous mixture equivalent to an approximately middle concentration of the calibration curve of brivudine and working concentration of ISTD during the start of the method validation and at the start of the respective day. The Area ratio of system suitability was within the acceptance limits of 5% CV.

**Method validation**

Method validation was done as per the criteria of industrial guidance for the bioanalytical method validation of USFDA [14-16].

**Carryover effect**

Carryover effect was performed in order to eliminate the carryover from the previous injection to the next injection. Extracted blank, extracted LLOQ, and extracted ULQQ samples were prepared from same biological matrix lot. These samples were injected in the order of mobile phase, extracted blank plasma, extracted LLOQ, extracted ULQQ and extracted blank plasma samples during the start of the method validation. The response of interfering peaks at the retention time of analyte has ≤ 20% of the response of extracted LLOQ and at the retention time of ISTD have ≤ 5% of response of extracted LLOQ.

**Selectivity/Sensitivity**

The selectivity of the method was assessed by analysing human blank plasma samples as of different healthy human volunteer’s six lots from a normal plasma source, two lots from lipemic source and two lots from haemolysed source, to investigate the potential interferences of endogenous compounds at the LC peak region for brivudine and ISTD. The selectivity of the method was determined with the LLOQ level of the analyte with PandA. The peak area of LLOQ for brivudine and ISTD at corresponding retention time in blank samples should not be more than 20% and 5% of the mean peak area of sofosbuvir.

**Linearity**

Linearity of the plasma samples was quantified using the peak-area ratio versus analyte nominal concentration. A calibration curve was constructed using linear regression and it consists of a standard blank sample, a standard Zero sample (Blank sample+ISTD) and eight non-zero samples which are distributed throughout the calibration range 85.205–4500.246 ng/ml for brivudine. The acceptance limit for a calibration curve was a correlation coefficient (r$^2$) of 0.98 or Greeter, and each back-calculated standard concentration should be ±5% deviation from the nominal value except LLOQ which was set at ±20%.

**Precision and accuracy**

Precision and Accuracy batch was assessed by analyzing three batches. For P and A studies five concentration QC samples were prepared as (LLOQ) Lower Limit of Quantification, (LQC) Lower Quality Control, (MQC) Medium Quality Control, (HQC) High-Quality Control and (DIQC) Dilution Integrated Quality Control, corresponding to 85.211, 240.030, 1500.188, 3500.204 and 17501.022 ng/ml respectively, quality control samples to the mean peak area of six non-zero samples which are distributed throughout the calibration range. These solutions were further diluted with a screened blank matrix without affecting the final concentration. The percentage recovery of ISTD from human plasma was determined by comparing the mean peak area of six extracted ITC, MQC and HQC with DIQC should be ≤ 15% except for LLOQ, which was set at ±20% and the intra- and inter-run accuracy for LLOQ, MQC, HQC and DIQC should be within ±15% except for LLOQ, which was set at within ±20%.

**Recovery**

The percentage recovery of brivudine from human plasma was determined by comparing the mean peak area of six extracted LQC, MQC and HQC corresponding to 240.030, 1500.188, 3500.204 and 17501.022 ng/ml respectively, quality control samples to the mean peak area of six post-extracted LQC, MQC and HQC quality control samples with the same concentrations.

The percentage recovery of ISTD from human plasma was determined by comparing the mean peak area of the prepared extracted ISTD to the mean peak area of Post extracted ISTD at the concentration level intended for use.

% recovery of ISTD has to be less than 10%.

**Dilution integrity**

Dilution integrity was assessed to ensure that samples could be diluted with a screened blank matrix without affecting the final concentration. Brivudine spiked human plasma samples were prepared at concentrations of 17501.022 ng/ml above the upper limit of the calibration range. These solutions were further diluted with human pooled plasma five times dilution in six replicates and analysed with PandA parameters. The six replicates have a precision of ≤ 15% CV and accuracy of 100±15%.
Ruggedness
The ruggedness of the method was studied by changing the experimental conditions such as concentration levels and analyst. The ruggedness results showed that the method was highly robust and reproducible.

Stock solution stability
The aqueous stock solutions were prepared and stored at 2-8 °C for 44 d. The mean peak response of the stored stock solution of analyte and ISTD was found to be within the acceptable range.

Bench-top stability
Stability of analyte was assessed by analyzing quality control samples at LQC and HQC concentration levels.

Long-term plasma stability
For long term stability, three sets of quality control samples (LQC and HQC) were stored in deep freezer at -20 °C for 44 d. The stability results showed that the method was highly robust and reproducible.

Freeze-thaw stability
Six replicates of stability quality control samples (LQC and HQC) were stored in deep freezer at -20 °C. Thawed at room temperature, followed by freezing for minimum 12hr. The QC samples were subjected to five freeze-thaw cycles before being extracted and analysed.

Auto-sampler stability
Six replicates of stability quality control samples (LQC and HQC) were processed and left in the auto-sampler rack for 16 h 30 min at 4 °C and then injected and quantified.

Wet extract stability
Six replicates of stability quality control samples (LQC and HQC) were processed and stored in wet extract form at 2-8 °C in the refrigerator for 24h.

RESULTS AND DISCUSSION
System suitability
The system suitability %CV of the retention time was found to be 0.41-1.55% for the analyte and 0.33-2.61% for ISTD. The % CV of the peak area ratio was found to be 2.33-4.10%. The results were found to be within tolerance and are presented in table 1.

Carryover effect
The result observed that no carryover was found during this method for both analyte and ISTD and it does not affect the precision and accuracy. The result was summarized in table 2.

Selectivity/sensitivity
The selectivity of the method was demonstrated on ten blank plasma samples obtained from different lots. The chromatograms were free from significant endogenous interferences. These batches were further selected for the preparation of standard QC and QC samples and the % bias of LLOQ samples was within the acceptable range of ±20%.

Linearity
The linearity of the method was demonstrated for brivudine in the concentration range of 85.205-4500.246 ng/mL. The correlation coefficients (r²) for the standard curves ranged from 0.9989 to 0.9997 for brivudine. The result was summarized in table 3.

Precision and accuracy
The intra-run and inter-run accuracy for each concentration level was within the range of 97.55-105.37% and 92.27-102.15%, respectively, and the intra-run and inter-run precision for each concentration level within the range of 2.99-6.31 %CV and 3.67-5.80 % CV, respectively. The intra-run and inter-run precision and accuracy of the method are presented in table 4.

Recovery
The mean % recovery for LQC, MQC, and HQC samples of brivudine were found to be 78.74%, 74.08%, and 67.50%, respectively. The mean % recovery of analyte was found to be 73.44% with acceptable precision of 7.69% CV and the ISTD % mean recovery was found to be 69.73% at MQC level. The result was summarized in table 5.

Dilution integrity
Dilution integrity of brivudine was performed and evaluated. The percentage nominal was found within the limit of ±15% and the estimated precision was ±15% and an accuracy of 100±15%. It shows that the drug can be diluted to five times and the results will be reproducible and are presented in table 4.

Stability
The % bias of stock solutions stability was tested for analyte and ISTD and established under refrigerator condition 2-8 °C for 44 d and results were found to be within the range of ±10%. The analyte passed all stability parameter tests viz., Auto-sampler (16 h 30 min), Bench-top (8h 30 min), wet extract (24h), Freeze-Thaw (5 cycles) and deep freezer stability at -20 °C for at least 44 d. There was no significant decrease in the analyte concentration was observed with a good percentage of accuracy and precision for stability quality control samples. The summary of the stability parameter for brivudine presented in table 7.

The developed chromatographic method for the quantification of brivudine in human plasma was simple, reproducible and economical, as exposed by the statistical data of analysis. The internal standard used is readily available. The mobile phase is simple to prepare and cost-effective. The analytical assay of brivudine was completed within 10.0 min, revealing the rapidity of the proposed RP-HPLC method. The linearity results showed that an excellent correlation existed between peak area and concentration of brivudine within the concentration range tested. The values of slope and intercept of the calibration graph indicated the good reproducibility of the proposed method. Higher values of correlation coefficient validated the linearity of the calibration plot. PandA results over three analytical batches are meeting the acceptance criteria. Overall mean recovery % CV for three QC levels less than 15.00%. Recovery statistical data was found to be within limits. Brivudine was found to be stable as long term shock at -20 °C for 44 d. When compared to previous bio-analytical methods [9-13], the developed method was simple, economical and accurate bio-HPLC method in human plasma.
Table 1: System suitability

| S. No. | Brivudine (Analyte) | Sofosbuvir (ISTD) | Area ratio |
|--------|---------------------|-------------------|------------|
|        | Retention time (min) | Retention time (min) |            |
| 1      | 3.57                | 7.88              | 0.3144     |
| 2      | 3.52                | 7.80              | 0.3230     |
| 3      | 3.55                | 7.91              | 0.2944     |
| 4      | 3.56                | 7.85              | 0.3767     |
| 5      | 3.58                | 7.92              | 0.3142     |
| mean±SD| 3.51                | 7.85              | 0.3003     |
| ±SD    | 0.0279              | 0.0445            | 0.0115     |
| %CV    | 0.79                | 0.57              | 3.74       |

*Number of injections-6

Table 2: Carry over effect

| Sample name       | Analyte area | % Carryover (analyte) | ISTD area | % Carryover (ISTD) |
|------------------|--------------|-----------------------|-----------|--------------------|
| Briv-Mobile Phase| 0            | 0.00                  | 0         | 0.00               |
| Briv-Extracted Blank| 0     | 0.00                  | 0         | 0.00               |
| Briv-Extracted LLLOQ | 3251 | N/AP                  | 184527    | N/AP               |
| Briv-Extracted ULOQ | 159468 | N/AP                  | 176238    | N/AP               |
| Briv-Extracted Blank| 0      | 0.00                  | 0         | 0.00               |

*Number of each concentration injections-3

Table 3: Linearity

| STD ID | STD 1 | STD 2 | STD 3 | STD 4 | STD 5 | STD 6 | STD 7 | STD 8 |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|
| n      | 3     | 3     | 3     | 3     | 3     | 3     | 3     | 3     |
| mean±SD| 85.798| 164.098| 355.289| 612.077| 973.657| 1688.178| 2851.115| 4627.626|
| ±SD    | 3.33  | 3.61  | 7.15  | 16.91 | 55.09 | 86.55 | 102.07 | 138.22 |
| %CV    | 3.88  | 2.20  | 2.01  | 2.76  | 5.66  | 5.13  | 3.58   | 2.99   |
| % Accuracy | 100.70| 96.30| 104.45| 104.95| 101.70| 105.26| 104.20| 102.83|

*Number of each concentration injections-3

Table 4: back-calculated concentrations for quality control samples (Intra-run and inter-run precision and accuracy)

| PA No. | LLOQ 85.211 ng/ml | LQC 240.030 ng/ml | MQC 1500.188 ng/ml | HQC 3500.204 ng/ml | DIQC 17501.022 ng/ml |
|--------|-------------------|-------------------|-------------------|-------------------|---------------------|
| n      | 6                 | 6                 | 6                 | 6                 | 6                   |
| Intra-run mean±SD | 87.170 | 235.249 | 1580.80 | 3560.44 | 18271.84 |
| Intra-run % Accuracy | 102.30 | 98.01 | 105.37 | 101.72 | 104.40 |
| n      | 6                 | 6                 | 6                 | 6                 | 6                   |
| Intra-run mean±SD | 85.704 | 234.159 | 1525.753 | 3475.64 | 17965.238 |
| Intra-run % Accuracy | 100.58 | 97.55 | 101.70 | 99.30 | 102.65 |
| n      | 6                 | 6                 | 6                 | 6                 | 6                   |
| Intra-run mean±SD | 88.268 | 245.448 | 1488.788 | 3430.830 | 17299.037 |
| Intra-run % Accuracy | 103.59 | 102.26 | 99.24 | 98.02 | 98.85 |
| n      | 18                | 18                | 18                | 18                | 18                  |
| Inter-run mean±SD | 87.047 | 238.286 | 1531.780 | 3488.971 | 17845.371 |
| Inter-run % Accuracy | 102.15 | 99.27 | 102.11 | 99.66 | 101.97 |

*Number of each concentration injections-6

Table 5: Recovery

| QC ID | LQC | Extracted area | MQC | Extracted area | HQC | Extracted area | ISTD | Extracted area |
|-------|-----|----------------|-----|----------------|-----|----------------|------|----------------|
| n     | 6   | 6              | 6   | 6              | 6   | 6              | 6    | 6              |
| mean±SD | 10990 | 8653   | 78700 | 58300 | 206637 | 139485 | 244963 | 170808 |
| ±SD   | 395  | 351            | 2603 | 3391          | 7286 | 9517           | 10887 | 5324          |
| %CV   | 3.59 | 4.06           | 3.31 | 5.81          | 3.53 | 6.82           | 4.44  | 3.12           |
| % Recovery | 70.74 | 74.08 | 67.50 | 69.73 | 73.44 | -              | -    | -              |

*Number of injections-6
Table 6: Ruggedness

| PandA   | LLOQ   | LQC    | MQC    | HQC    | DIQC   |
|---------|--------|--------|--------|--------|--------|
|         | 85.211 | 240.030| 1500.188| 3500.204| 17501.022 |
| n       | 6      | 6      | 6      | 6      | 6      |
| Intra-run mean±SD | 79.260 | 227.333| 1529.991| 3583.002| 17548.005 |
| Intra-run SD    | 1.58   | 9.63   | 71.93  | 198.80 | 822.62 |
| Intra-run %CV  | 2.00   | 4.24   | 4.70   | 5.55   | 4.69   |
| Intra-run % Accuracy | 93.02 | 94.71 | 101.99 | 102.37 | 100.27 |

*Number of each concentration injections-6

Table 7: Stability

| Stability experiment | QC ID | Nominal concentration (ng/ml) | Concentration found (ng/ml) (mean±SD)* | Precision (% RSD) | Accuracy (%) |
|----------------------|-------|------------------------------|----------------------------------------|-------------------|-------------|
| Benchtop Stability   | LQC   | 240.030                      | 247.259                                | 5.28              | 103.01      |
| Stability            | HQC   | 3500.204                     | 3410.719                               | 7.72              | 97.44       |
| Freeze-thaw Stability| LQC   | 240.030                      | 244.894                                | 7.39              | 102.03      |
| Stability            | HQC   | 3500.204                     | 3515.815                               | 4.45              | 100.45      |
| Wet extract Stability| LQC   | 240.030                      | 238.023                                | 3.36              | 99.16       |
| Stability            | HQC   | 3500.204                     | 3574.314                               | 5.24              | 102.12      |
| Autosampler Stability| LQC   | 240.030                      | 248.148                                | 4.65              | 103.38      |
| Stability            | HQC   | 3500.204                     | 3498.503                               | 4.12              | 99.95       |
| Long term plasma stability | LQC | 240.030                      | 234.321                                | 4.26              | 97.62       |
| Stability            | HQC   | 3500.204                     | 3565.980                               | 4.72              | 101.88      |
| DIQC                 |       | 17501.022                    | 17115.229                              | 3.86              | 97.80       |

*Number of each concentration injections-6

Fig. 2: Extracted LLOQ of brivudine

Fig. 3: Extracted ULOQ of brivudine
CONCLUSION
A simple, accurate, precise, linear and rugged bioanalytical HPLC method for the brivudine was developed and validated with sofosbuvir used as ISTD. The developed method was validated according to the USFDA regulatory guidelines procedure (May-2018) and all the validation parameters were within the acceptable range. Hence, the method can use for application in bioavailability/bioequivalence (BA/BE) studies and Therapeutic Drug Monitoring (TDM) with desired precision and accuracy along with high throughput.

FUNDING
Nil

AUTHORS CONTRIBUTIONS
Both author have contributed equally.

CONFLICT OF INTERESTS
Declared none

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