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VALIDATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR DETERMINATION OF STAVUDINE IN HUMAN PLASMA

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Abstract

A simple, accurate, precise and sensitive HPLC method with UV detection was developed and validated to estimate Stavudine in human plasma. It was extracted from human plasma using methanol, by using protein precipitation method. It was chromatographed on a Phenomenex Luna C_{18} column using 50µL injection volume and UV detector. An isocratic mobile phase consisting of a mixture of ortho phosphoric acid buffer (pH 3.8) and Methanol (1:9) was used in the analysis. The retention time (R_t) of the drug was found 6.5 mins. The method was validated over the range of 0.025µg/mL to 1.750 µg/mL. The intra and inter-day precision (%RSD.) values of Stavudine for various concentrations ranged from 1.369% to 4.166%. The maximum recovery was achieved at medium and high quality control samples and was 98.40%. The described method can be readily utilized for routine quality control and evaluation of clinical data of Stavudine in human plasma.

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INTRODUCTION

Different analytical methods are employed for the quantitative determination of drugs and their metabolites in biological samples. These methods play a significant role in the interpretation of bioavailability, bioequivalence and pharmacokinetic data, in various studies. Thus it is essential to employ well characterized and fully validated analytical methods which can yield reliable results [1]. Stavudine is a synthetic nucleoside analogue with activity against HIV-1 and HBV [2].

The chemical name of Stavudine is 2', 3'-didehydro-3'-deoxythymidine. It has a molecular formula of $C_{10}H_{12}N_2O_4$ and a molecular weight of 224. Stavudine is a white or almost white powder. It is freely soluble in Ethanol (95%) and sparingly soluble in Water. Its solubility at 23°C is approximately 83 mg/mL in water and 30 mg/mL in propylene glycol. The drug is officially listed in monograph of USP & I.P. [3, 4]. Several analytical methods that have been reported for the estimation of Stavudine in biological fluids or pharmaceutical formulations, which include High Performance Liquid Chromatography, Titrimetry and UV-Visible Spectrophotometry [5-16]. These methods used different process for separating the protein from plasma. Protein precipitation technique for sample preparation involves fewer steps, but during sample analysis column is often blocked and high back pressure is witnessed. Also sample filtration in protein precipitation method is very difficult and expensive. Also in protein precipitation process, a guard column is required to protect and avoid damage to the column which is expensive. But in liquid-liquid extraction process sample is very clean and no column damage was occurring as well as it is cost effective. The main advantage of this method is the use of liquid-liquid extraction procedure for preparation of plasma. It is easy and faster than the reported methods. No such method has been published for quantification of Stavudine by HPLC with UV detection in human plasma by using liquid extraction. So, this method may be used as a reliable and simpler assay method.

MATERIALS AND METHOD

Materials and Reagents: Stavudine (STV) (purity ≥ 99%) bulk drug was obtained from Acme formulation Pvt. Ltd., (Himachal Pradesh, India). KH_2PO_4 (99%) (Analytical-

reagent grade) and methanol (HPLC- grade) were purchased from Merck Pvt. Ltd. (Mumbai, India). HPLC grade water, generated from Milli Q water purification system was used throughout the analysis. The blank human plasma with EDTA-2K anticoagulant was collected from Clinical Pharmacological unit (CPU) S.C.B medical college, Cuttack. All other reagents used were of analytical grade (Merck Pvt. Ltd. Mumbai, India).

Apparatus and Chromatographic Conditions: Analyses were performed on Shimadzu scientific instruments composed of LC-20 AT pump and SPD-20 AT variable wavelength detector. The separation of compounds was achieved using a Phenomenex Luna C_{18} column (5µm, 25cm X 4.6mm i.d). A mixture of ortho phosphoric acid buffer (pH 3.8) and methanol was used in the ratio of 1:9 in the analysis. It was filtered through 0.45µm membrane filter. A flow rate of 0.8 mL/min and a sample size of 50µL were injected through the rheodyne injector.

Preparation of Stock and Standard Solution: The stock solutions of 1 mg/mL of the analyte STV were prepared in methanol, respectively. These solutions were further diluted suitably with the mobile phase to obtain a stock solution which was further used to prepare the working standard solution for calibration curve and quality control (QC) samples. A calibration curve was prepared by spiking appropriate amounts of working solution into the blank plasma to obtain final concentrations of 0.025, 0.050, 0.100, 0.250, 0.500, 1.000, 1.500 and 2.000 µg/mL for the analyte. The QC samples were also prepared in the same way to obtain the final concentrations of 0.075 (low QC), 1.250 (mid QC) and 1.800 (high QC) µg/mL. All stock solutions and working standard solutions were stored in polypropylene vials in a -20°C freezer.

Preparation of Calibration and Quality Control Samples: Liquid- liquid extraction procedure was used for the extraction of the Stavudine from the plasma. For calibration standards and quality control samples, an aliquot quantity of 0.5mL plasma sample was taken in a 10mL stopper test tube. To it, 4.5mL of methanol was added and mixed for 15 minutes followed by centrifugation at 5000 rpm for 20 min. The organic layer was separated, transferred to a separated test tube and

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evaporated to dryness under the stream of N_2 at 40°C. The residue was reconstituted in 200µL of mobile phase, filter through 0.22µm membrane filter and 50µL was injected into the HPLC system. For calibration standards, an aliquot of 50µL of working stock solution of Stavudine was spiked into 500µL of blank plasma in poly propylene tube. Samples for the determination of recovery, precision and accuracy were prepared by spiking the blank plasma with the analytes at lower limit of quantitation (LLOQ), low quality control (LQC), mid quality control (MQC) and high quality control (HQC) concentrations into different tubes. The samples for the stability studies were also prepared in the same way at appropriate concentrations (LQC, MQC and HQC) into different tubes and were stored at -20°C until analysis.

Method Validation: The method was validated as per the criteria of industrial guidance for the bioanalytical method validation.

Linearity and LLOQ: A series of calibration standards were prepared by adding a known concentration of Stavudine to drug free human plasma and analyzed. The lowest concentration on the standard curve with detector response five times greater than the drug free (blank) human plasma was considered as the LLOQ. The analyte peak in LLOQ sample should be identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80 to120%. The results are shown in the Table: 1.

Specificity and Selectivity: It was done by analyzing the blank plasma samples from six different sources to for any chromatographic interference at the retention times of the analyte. Representative chromatograms of blank plasma, blank plasma spiked with STV is shown in the Fig 1 (a) and (b). Run time of the chromatogram was 10 min and the retention time of the drug was about 6.5 min, respectively. No interfering peaks were found in the chromatogram obtained from blank plasma.

Precision and Accuracy: Three validation batches were processed on three different days. Each batch included six replicates of each LLOQ, low-, medium- and high-concentrations of QC samples. The results from QC samples in six runs were used to

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evaluate the accuracy and precision of the method. Accuracy was calculated by the ratio of determined concentration and actual concentration multiplied by 100 %, and precision was evaluated by the percentage relative standard deviations (% RSD). The criteria for acceptability of the data included accuracy within ±15 (% RSD) from the nominal values and a precision of within ±15 (%RSD), except for LLOQ, where it should not exceed ±20% of SD. The results of accuracy and precision data of the analysis in Human plasma are given in the Table 2.

Recovery Studies: A Recovery study of analyte was determined by measuring the peak areas of the drug from the prepared plasma quality control samples. 0.075, 1.250 and 1.750 µg/mL were taken as LQC (low quality control), MQC (medium quality control) and HQC (high quality control) samples, respectively. The peak areas of extracted LQC, MQC and HQC were compared to the absolute peak area of the un-extracted samples containing the same concentration of the drug as 100%.

Stability study: The stability of the analytes after three freeze and thaw cycles was determined at low, middle, high QC samples. The samples were stored at -20°C for 24 hour and thawed unassisted at room temperature. After completely thawing, the samples were refrozen for 12-24 h. After three freeze- thaw cycles, the concentration of the samples were analyzed. For ambient and bench top stability study the QC samples were kept at room temperature for 8 h and 24 h respectively, after which they were extracted and analyzed. Stability was concluded when the % RSD was within ±15% of the actual value. Six aliquots of each low, middle and high QC samples were kept in deep freezer at -20±5°C for one month and three months. The samples were processed and analyzed and the concentrations obtained were compared with the actual value of QC samples to determine the short term stability and long term stability of the analyte in human plasma. Samples were concluded stable if the %RSD of the stability samples was within ±15% of the actual value. The results are given in the Table 3.

RESULTS AND DISCUSSION

Linearity and LLOQ: Initially, validation was performed to evaluate the calibration,

accuracy and precision of Stavudine in human plasma. The calibration curve was constructed by plotting the peak area versus the nominal concentration (C) of the analyte. The plasma calibration curves were linear ($r2$>0.999) over the concentration range of 0.025- 2.000µg/mL. The standard curve had a reliable reproducibility over the standard concentrations across the calibration range.

Accuracy and precision: The intra- and inter-day precision (%RSD) values of Stavudine for various concentrations ranged from 2.091% to 1.506% and 2.09% to 1.364 % respectively. At the same concentrations, the percentage of accuracy was in the range of 96%-97.14% and 96%-98.28 % respectively. Both accuracy and precision were in the acceptable range for bioanalytical purpose.

Extraction recovery: Recovery results presented that the maximum recovery was achieved at medium-(1.250µg/ml) and high (1.750µg/ml) quality control samples was 98.40%. The extraction recovery was found to be satisfactory as it was consistent, precise and reproducible.

Stability study: Each stability test included six replicates of three levels of QC samples. In human plasma, Stavudine was stable for three freeze/thaw cycle to three month frozen condition at -20°C. The experimental data of the stability tests, i.e. 24 h ambient temperature, bench-top (8 h), three repeated freeze- thaw cycles and at -20°C for 30 days, 90 days respectively showed that predicted concentrations for analyte at LQC, MQC and HQC samples deviated within the assay variability limits (±15%) of the nominal concentrations.

CONCLUSION

The proposed HPLC method is simple, isocratic, rapid, specific, accurate and precise for the Stavudine determination of in bulk and human plasma. Hence, it can be recommended for the routine quality control and evaluation of clinical data of Stavudine.

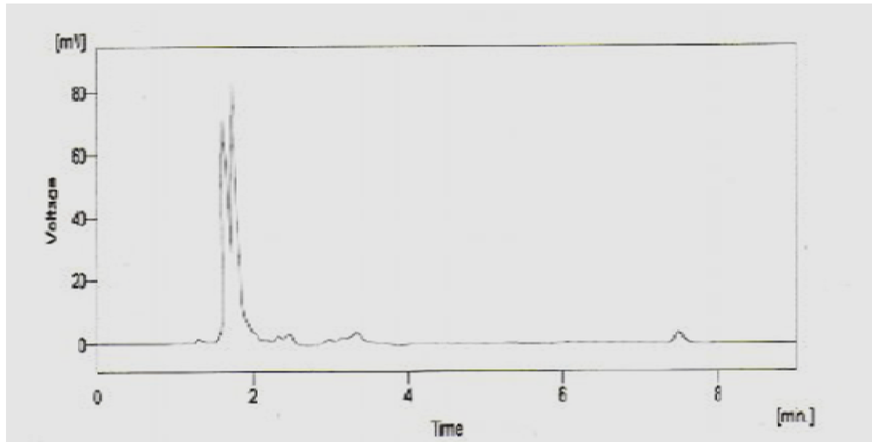

Figure 1 (a) Representative chromatogram of Blank human plasma

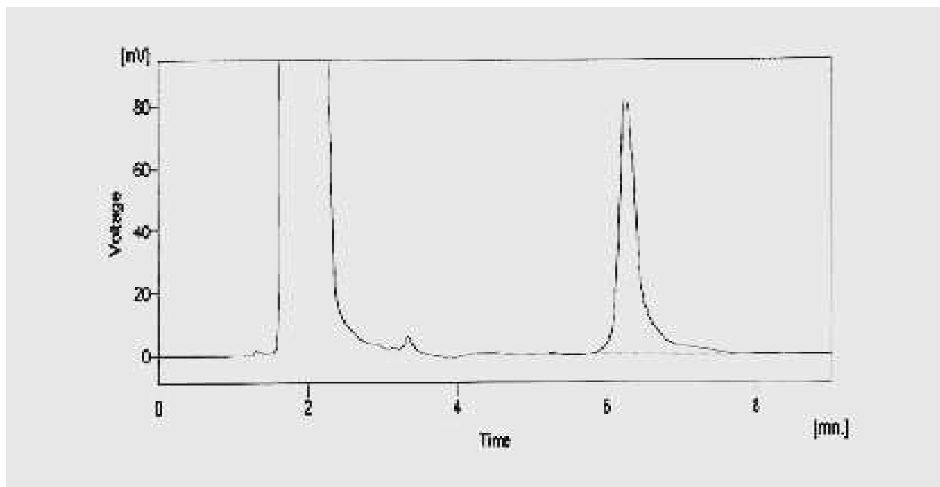

Figure 1 (b) Representative chromatogram of Stavudine in human plasma

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Table 1

Linear Regression data for the Determination of Stavudine added in Human Plasma

Spiked Concentration (ppm)	**Area**
0.025	100777
0.05	300428
0.1	409976
1.5	5929940
1.75	6969592
2	8539520
Correlation coefficient	0.999

Table 2

Accuracy and Precision data of the Analysis in Human plasma (n= 6)*

Intraday						**Interday**			
Spiked conc. (µg/ml)		Mean	SD	Accuracy %	% RSD	Mean	SD	Accuracy%	% RSD
LLOQ	0.025	0.024	0.0005	96.00	2.091	0.024	0.0005	96.00%	2.091
LQC	0.075	0.073	0.0018	97.33%	1.369	0.074	0.0011	98.66%	1.576
MQC	1.25	1.231	0.0173	98.40%	1.408	1.234	0.0161	98.40%	1.304
HQC	1.750	1.707	0.0257	97.14	1.506	1.729	0.0236	98.28%	1.364

*Accuracy (%), [(measured concentration/spiked concentration) x 100]; SD, Standard deviation; RSD (%), relative standard deviation [(S.D./mean) x 100]; LLOQ ,lower limit of quantification; LQC, low quality control; MQC, middle quality control; HQC, high quality control ; n = number of sample

Table 3

Stability Data of Stavudine in Human Plasma (n = 6)*

Storage condition	LQC (0.050 µg/mL)	MQC (1.25µg/mL)	HQC (1.75 µg/mL)
3 Freeze-thaw cycle	96.822±1.591	95.513±1.711	97.188±0.639
24 h ambient	93.438±1.006	96.34483±1.001	94.905±0.789
8 h bench top	94.522±0.866	96.531±1.736	94.709±1.464
1 month frozen (-20 °C)	92.920±1.390	92.671±1.219	92.015±0.943
3 months frozen (-20 °C)	90.703±0.806	91.766±0.675	91.722±1.042

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