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Quantification of Etravirine in Rat Plasma by LC-MS/MS and Application to a Pharmacokinetic Study

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Authors' contributions

This work was carried out in collaboration between all authors. Authors VMB and KB designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors BRC and KB managed the literature searches, analyses of the study performed the spectroscopy analysis and author MA managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

A rapid, rugged and reproducible, higher in sensitivity bio-analytical method was developed for quantification of Etravirine (EV) in rat plasma by LC-MS/MS. Etravirine $^{15}N_2$, $^{13}C_1$ (EVIS) used as an internal standard (IS). Chromatography was performed with ZORBAX Eclipse Plus Phenyl-Hexyl (50 mm × 2.1 mm × 3.5 μm) analytical column. Mobile phase was composed with 0.1% formic acid:

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acetonitrile (45:55 v/v), at a flow rate of 0.3 ml/min. Product ions of EV (163.1) and EVIS (166.1) were formed from the parent ion of EV (436.1) and EVIS (440.1). The drug and the IS were extracted from Liquid-liquid extraction (LLE) method. The calibration range is 5.0–750.0 ng/ml with a coefficient of determination (R^2) is greater than 0.9950. This method demonstrated intra and inter day precision within 1.38 – 2.26% and 1.32–2.75%, and an accuracy within 99.77 – 100.80%, and 99.50 – 102.15%. Stability of EV in rat plasma was proved for freeze-thaw cycles, benchtop, and long term and Autosampler conditions. Pharmacokinetics study was studied in 6 healthy rats.

Keywords: Etravirine; rat plasma; validation; pharmacokinetic study; LC-MS/MS.

1. INTRODUCTION

Etravirine is used for the treatment of human immunodeficiency virus type 1 (HIV-1) infection. Chemically, it is 4-({6-amino-5-Bromo-2-[(4-cyanophenyl) amino] pyrimidin-4-yl} Oxy)-3, 5-dimethylbenzonitrile. Molecular weight is about 435.28 [1] (Fig.1).

Maximum oral absorption is achieved in 2.5 - 4 hr [2]. Plasma protein binding is about 99.9%. Etravirine is metabolized by the liver and 93.7% was found to undergo fecal elimination 1.2% of the dose was renally eliminated, changed [3,4]. Etravirine half life is about 9.05- 41 hours. Renal clearance of etravirine is negligible (<1.2%), thus no dose adjustments are required in patients with renal impairment [5].

As of now, a few methods were reported for quantification of EV in rat plasma with LC-MS, [6] human plasma by LC-MS, [7-12] human blood with LC-MS [13], Human plasma by HPLC, [14, 15]. The developed method [6] in rat plasma has some drawbacks in terms of sensitivity, ruggedness and reproducibility.

The aim of the proposed method is to develop rapid, rugged and reproducible method and extract the EV in rat plasma by the LLE method with high sensitivity. Moreover, the analyte is to be compared with the deuterated internal standard (IS), which is most appropriate to develop in the Bioanalytical method.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

EV and EV $^{15}N_2$, $^{13}C_1$ were procured from Clear Synth, India. Formic acid (analytical grade) was purchased from Merck, Mumbai, India. Acetonitrile, methyl t-butyl ether (HPLC grade) were obtained from J. T. Baker, USA. Rats were obtained from Bioneeds, Bangalore.

2.2 Instrumentation

High Precision Liquid Chromatography (HPLC) system with 1200 series from Agilent technologies was used. Triple quadrupole Mass spectrometer 4000 model was used from ABI-SCIEX, Toronto, Canada. Data processing was obtained from Analyst 1.5.1 software package (SCIEX).

2.3 Detection

Detection was by turbo ion spray (API) positive mode with unit resolution. For EV, mass transitions were obtained from 436.1 m/z (parent ion) to 163.3 m/z (product ion). Similarly,

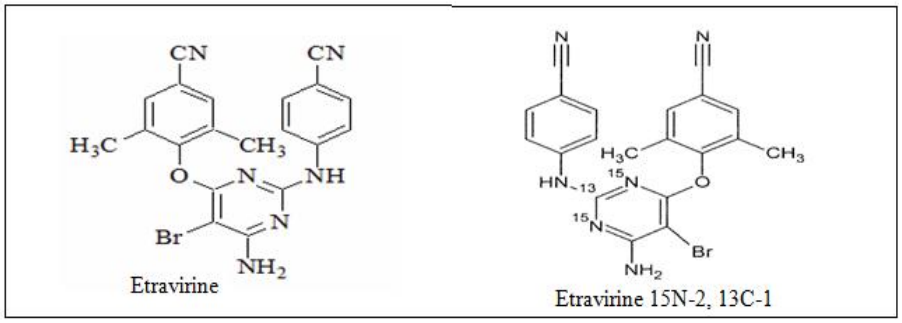

Fig. 1. Chemical structures of Etravirine and Etravirine 15N-2, 13C-1

$EV^{15}N_2$, $^{13}C_1$ mass transitions were obtained from (440.1 m/z) (parent ion) to 166.1 m/z (product ion).

2.4 Chromatographic Conditions

Chromatographic separation was performed using an ZORBAX Eclipse Plus Phenyl-Hexyl column (50 mm × 2.1 mm ×3. 5 μm) at a temperature of 30°C. The mobile phase was composed of 0.1% formic acid: acetonitrile (45:55 v/v) at a flow rate of 0.3 ml/minute. Etravirine $^{15}N_2$, $^{13}C_1$ was used as the appropriate IS in terms of chromatography and extractability. EV and EVIS were eluted at 1.6±0.2 minutes, approximately, with a total run time of 2.2 minutes for each sample.

2.5 Preparation of Standards and Quality Control Samples

Standard stock solutions of EV (200.0 µg/ml) and EVIS (100.0 µg/ml) were prepared in acetonitrile. The IS spiking solutions (1250.0 ng/ml) were prepared in 60% acetonitrile solution from IS stock solutions. Standard stock solutions and IS spiking solutions were stored in refrigerated conditions for 2–8°C until analysis. Standard stock solutions of EV (200.0 µg/ml) were added to drug-free screened Rat plasma to obtain concentration levels of 5.0, 10.0, 25.0, 75.0, 150.0, 300.0, 450.0, 600.0 and 750.0 ng/ml for analytical standards, and 5.0, 15.0, 375.0, and 525.0 ng/ml for quality control (QC) standards, and stored in the freezer at –30°C until analysis. The aqueous standards were prepared in a reconstitution solution (0.1% formic acid: acetonitrile (45:55 v/v)) and stored in the refrigerator at 2–8°C until analysis.

2.6 Sample Preparation

The LLE method was used to isolate EV and EVIS from Rat plasma. For this purpose, 50 µL of EVIS (1250 ng/mL) and 50 µL of plasma sample concentration, were added to labeled polypropylene tubes and vortexed briefly for about 5 minutes. Thereafter, 2.5 mL of extraction solvent (Methyl t-butyl ether (TBME) was added and vortexed for about 10 minutes. Next, the samples were centrifuged at 4000 RPM for approximately 5 minutes at ambient temperature. From each, a supernatant sample was transferred into labeled polypropylene tubes and evaporated to a dryness of 40ºC briefly, and then reconstituted with a reconstitution solution ((0.1% formic acid: acetonitrile (45:55 v/v))), and the sample transferred into auto sampler vials and injected into the LC-MS for study.

2.7 Selectivity

Selectivity was performed by analyzing rat blank plasma samples from six different sources with an additional hemolyzed group and the lipedimic group to test for interference at the retention times of analyte.

2.8. Precision and Accuracy

Precision and accuracy were determined by six replicates of LLOQ, LQC, MQC and HQC. The precission should be less than 15%, and accuracy within 85-115%, except for LLOQ, where it should be 80-120%.

2.9 Matrix Effect

The matrix effect caused due to the plasma matrix was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (LLE) with the reconstitution samples extracted from the blank plasma sample spiked with the analyte. Experiments in triplicate were performed at LQC and HQC levels with 6 different plasma lots with the acceptable precision (% CV) of ≤15%.

2.10 Recovery

The extraction recovery (absolute recovery) of analyte and IS from rat plasma was determined by analyzing QC samples. Recovery (absolute recovery) at 3 concentrations (15.0, 375.0, and 525.0 ng/ml) was determined by comparing peak areas obtained from the plasma sample and the standard solution spiked with the blank plasma residue. A recovery (absolute recovery) of more than 50% was considered adequate to obtain the required sensitivity.

2.11 Stability (Freeze-thaw, Auto Sampler, Room Temperature, Long-term)

LQC and HQC samples (n = 6) were retrieved from deep freeze after 3 freeze-thaw cycles according to clinical protocol. Samples were stored at –30°C in 3 cycles of 24, 36, and 48 h**.** In addition, the long-term stability of EV in QC samples was also evaluated by analysis after 76 days of storage at –30°C. Auto-sampler stability was studied following a 65-h storage period in the auto sampler tray with control concentrations.

Room temperature stability was studied for 24 hr. The % CV should be ≤15 and ±15%.

2.12 Analysis of Rat Samples

The Bioanalytical method described above was used to determine EV concentrations in plasma following oral administration of EV tablet to healthy rats (n=6). Sprague-Dawley rats with weight between 200-350 g were selected for the study. The animals were kept in individual cages and maintained at 25°C for 10 days prior to the experiment. Standard diet and water *ad libitum* were given to them. All experiments have been performed according to guidelines of the Institutional Animal Ethics Committee, Albino research and training institute, Hyderabad. EV 1.35 mg/ 200 g (100 mg Human equivalent dose in suspension form) body weight was administered orally by oral gavage using an animal feeding needle in a single dose. All studies were performed after keeping rats for overnight fasting. Blood samples (0.3 mL) were colleted at predose and 0.25, 0.5, 1, 1.5, 2, 2.5, 5, 8, 12 and 24 h of post–dose in heparinized Eppendorf tubes. These samples were centrifuged immediately at 3500 RPM and 4°C temperature for 10 min. Plasma samples were taken and stored at −30°C until assay. The Pharmacokinetic parameters like peak plasma concentration (C_{max}) time to reach peak plasma concentration (T_{max}), area under the (concentration–time) curve (AUC) and elimination half–life ($t_{1/2}$) were calculated following non–compartment model of WinNonlin Version 5.1.

2.13 Pharmacokinetics and Statistical Analysis

Blood samples were taken during a period of 3–5 times the terminal elimination half-life ($t_{1/2}$), and it was considered as the area under the concentration time curve (AUC) ratio higher than 80%, as per FDA guidelines [16]. Plasma EV concentration-time profiles were visually inspected, and C_{max} and T_{max} values were determined. The AUC_{0-t} was obtained by the trapezoidal method [17,18].

3. METHOD DEVELOPMENT

During development of the method, different options were evaluated to optimize mass spectrometry detection parameters, chromatography, and sample extraction.

3.1 Mass Spectrometry Detection Parameters Optimization

The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at both polarities at a flow rate of 5 µL/min. EV gave a better response when in the positive ion mode as compared to the negative ion mode. The predominant peaks in the primary ESI spectra of EV and EVIS correspond to the $(M+H)^+$ ions at m/z 436.1 and 440.1, respectively (Figs. 2(A) and 2(c)). Product ions of EV and EVIS scanned in quadrupole 3 after a collision with nitrogen in quadrupole 2 had a m/z of 163.1 and 166.1, respectively (Figs. 2(B) and 2(D)). Mass parameters were optimized as source temperature 500°C, heater gas 45 psi (nitrogen), nebulizer gas 30 psi (nitrogen), curtain gas 20 psi (nitrogen), CAD gas 5 psi (nitrogen), ion spray (IS) voltage 5500 volts, source flow rate 300 µL/min without split, entrance potential 10 V, declustering potential 55 V, collision energy 25 V, and collision cell exit potential 12 V for both analyte and IS.

3.2 Chromatography Optimization

Initially, a mobile phase consisting of ammonium acetate and acetonitrile in varying combinations was tried, but a low response was observed. The mobile phase containing ammonium formate, methanol (20:80 v/v), gave a better response, but poor peak shape was observed. A mobile phase of 0.1% formic acid in water in combination with methanol and acetonitrile with varying combinations were tried. Finally, a mobile phase containing 0.1% Formic acid, acetonitrile (45:55 v/v), gave the best signal along with a marked improvement in the peak shape observed for EV and EVIS. Short-length columns, such as Symmetry Shield RP18 (50 × 2.1 mm × 3.5 µm), Inertsil ODS-2V (50 × 4.6 mm × 5 µm), Hypurity C18 (50 × 4.6 mm × 5 µm), and Hypurity Advance (50 × 4.0 mm × 5 µm), Xbridge C18 (50 × 4.6 mm 5 µm), ZORBAX Eclipse Plus Phenyl-Hexyl (50 × 2.1 mm 3.5 µm), were tried during development of the method.), ZORBAX Eclipse Plus Phenyl-Hexyl (50 × 2.1 mm 3.5 µm) column gave a relatively good peak shape with the best signal being obtained.

3.3 Extraction Optimization

LLE, SPE, PPT extraction methods were performed to extract of EV and EVIS from rat

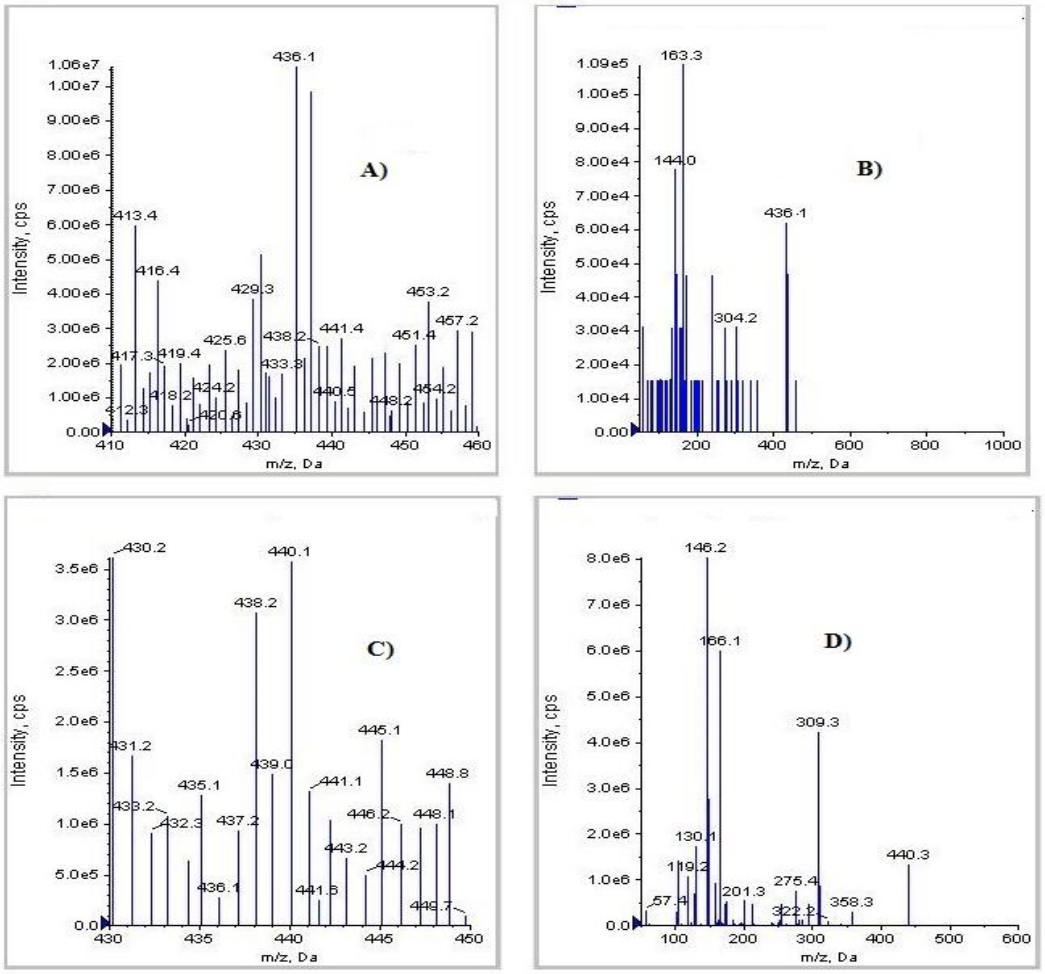

Fig. 2. Parent ion mass spectra of A) Etravirine parent ion B) Etravirine product ion C) Etravirine 15N-2, 13C-1 parent ion D) Etravirine 15N-2, 13C-1 product ion

plasma. Among all LLE with methyl tertiary butyl ether suitable for good recovery and response.

4. METHOD VALIDATION

The developed method was validated as per regulatory guidelines [16]. The experiments conducted under validation section are for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, and stability.

4.1 Selectivity

The analysis of EV and EVIS using MRM function was highly selective with no interfering compounds (Fig. 3a). Specificity was performed by using 6 different lots of Rat plasma. Chromatograms obtained from plasma spiked with EV (5.0 ng/ml) and EVIS (1250.0 ng/ml) are shown in Fig. 3c.

4.2 Matrix Effect

The overall precision of the matrix factor is expressed as CV% and was determined to be 0.80 at LQC and 0.83 at HQC level for the analyte.

4.3 Linearity

Calibration curves were plotted as the peak area ratio (EV/EVIS) *versus* (EV) concentration. Calibration was found to be linear over the concentration range of 5.0–750.0 ng/ml. The % CV was less than 3.73%, and the accuracy ranged from 98.52 to 102.69%. The determination coefficients (r^2) were greater than 0.9950 for all curves (Table 1).

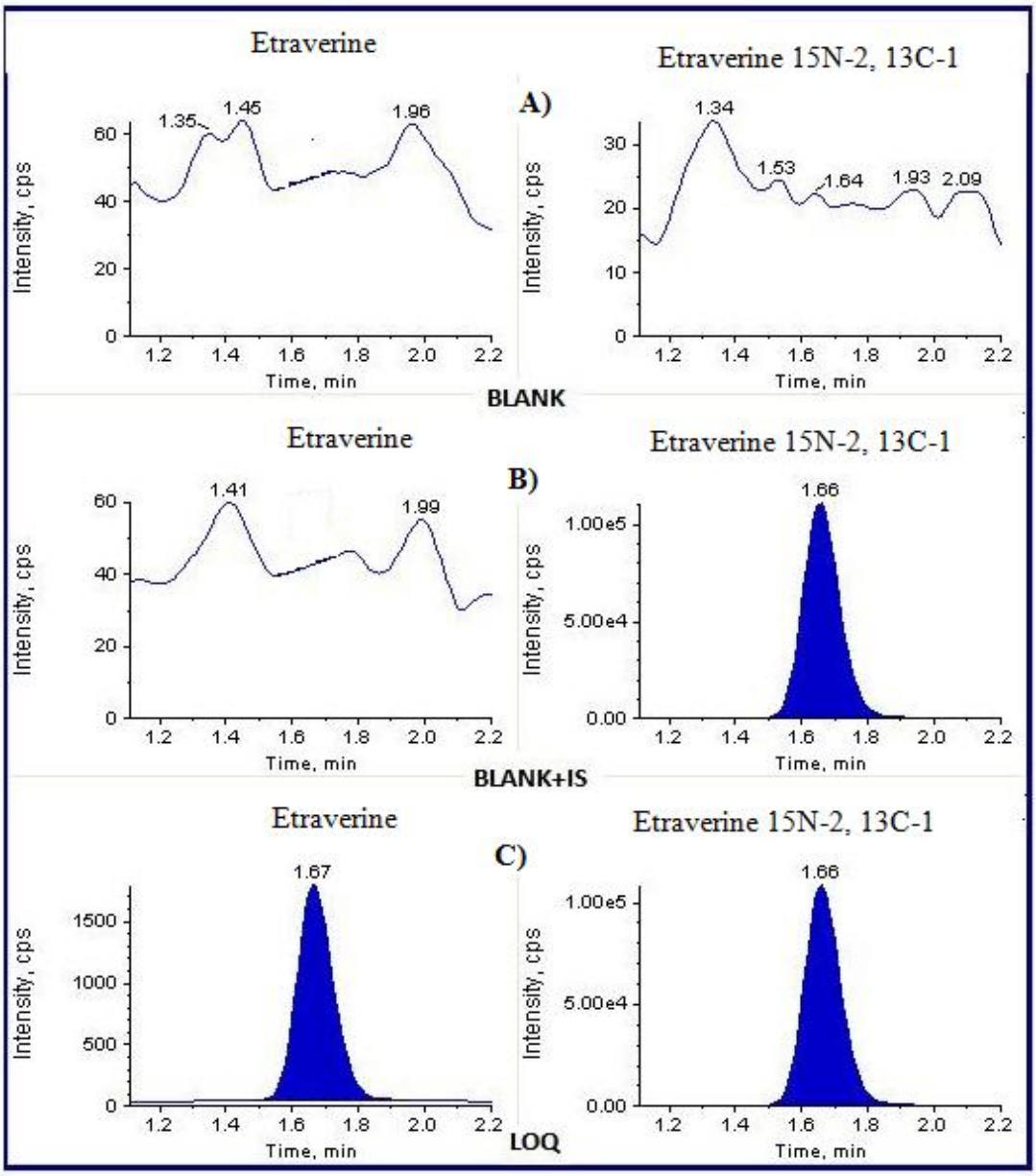

Fig. 3. Chromatogram of A) Blank B) Blank+IS C) LOQ for Etravirine and Etravirine 15N-2, 13C-1

4.4 Precision and Accuracy

Precision and accuracy for this method were controlled by calculating the intra- and inter-batch variations at four concentrations (5.0, 15.0, 375.0, and 525.0 ng/mL) of QC samples in 6 replicates. As shown in Table 2, the intra batch % CV was less than 2.26%, and the accuracy ranged from 99.77 to 100.80%. Inter batch % CV was less than 2.75%, and the accuracy ranged from 99.50 to 102.15%. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

4.5 Recovery

The recovery of EV was determined at three different concentrations of 15.0, 375.0, and 525.0 ng/ml, and were found to be 77.49, 86.97, and 91.67%, respectively. The overall average

recovery of EV and EV IS was found to be 85.38 and 87.08%, respectively.

4.6 Limit of Quantification (LOQ)

The LOQ was determined at 5 ng/ml.

4.7 Stability (Freeze-thaw, Auto sampler, Bench-top, Long-term)

Quantification of the EV in plasma, which was subjected to 3 freeze-thaw (–30°C up to room temperature) cycles showed the stability of the analyte. The concentrations ranged from 96.46 to 99.84% of the theoretical values. No significant degradation of the EV was observed even after a 79-h storage period in the auto sampler tray, and the final concentration of EV was between 106.89 and 106.41% of the theoretical values. Room temperature stability at 26 h was between 97.67 and 98.32% of the theoretical values. In addition, the long-term stability of EV in QC samples after 76 days of storage at –30°C was also evaluated. The concentrations ranged from 99.81 to 100.73% of the theoretical values. These results confirmed the stability of EV in Rat plasma for at least 76 days at –30°C (Table 3).

4.8 Application to Biological Samples

The above validated method was used in the determination of EV in plasma samples for establishing the pharmacokinetics of a single 200 mg/ kg (100 mg Human equivalent dose of tablets) weight in 6 healthy rats by oral route. Fig. 4 depicts the mean plasma concentration *vs* time profile of EV. All the plasma concentrations of EV were within the standard curve region and retained above the 5.00 ng/ml (LOQ) for the entire sampling period (Table–4).

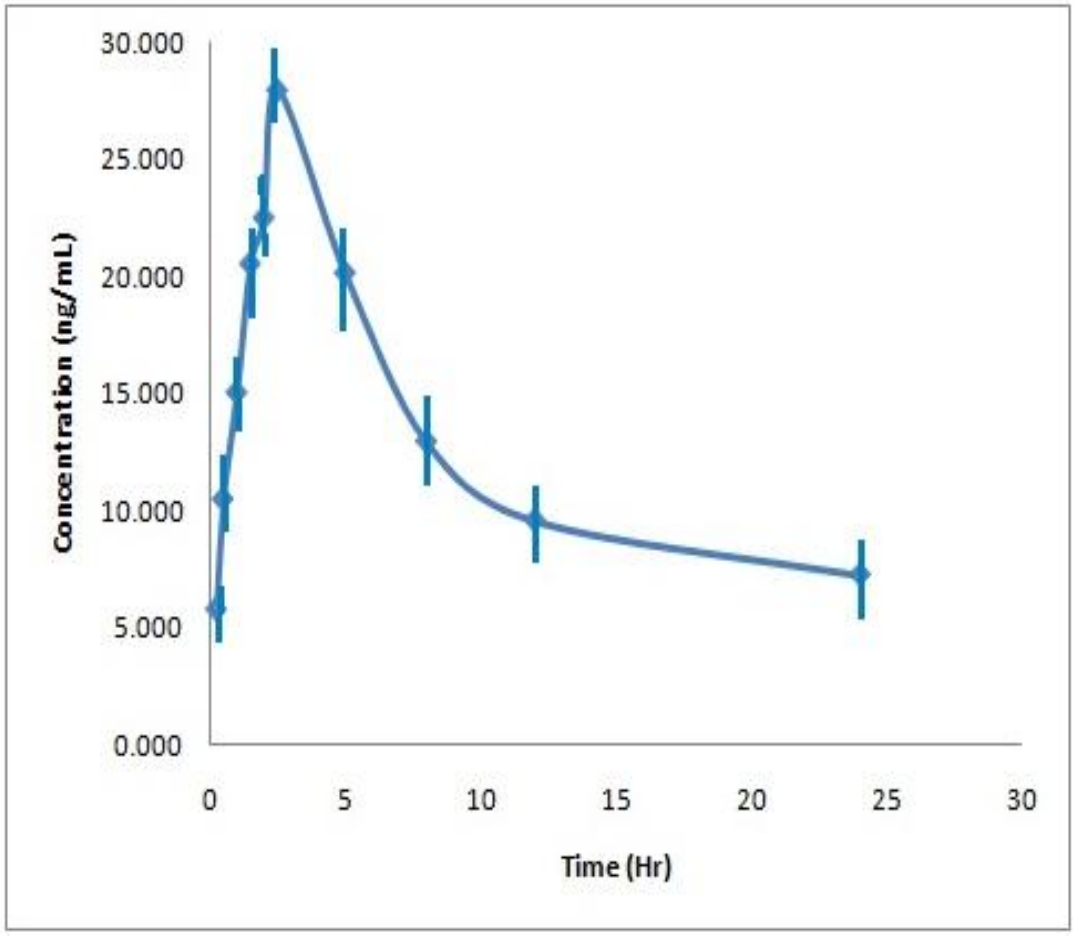

Fig. 4. Mean Plasma concentrations of Etravirine after oral administration (1.35 mg/200 g) dose

Table 1. Calibration curve details from one batch of the validation section

Spiked plasma concentration (ng/mL)	Concentration measured(mean) (ng/mL)	SD	CV (%) (*n* = 5)	Accuracy %
5.00	5.01	0.04	0.87	100.16
10.00	9.85	0.16	1.62	98.52
25.00	25.67	0.36	1.4	102.69
75.00	76.08	1.25	1.64	101.44
150.00	148.02	1.34	0.9	98.68
300.00	292.68	3.70	1.26	97.56
450.00	456.02	5.02	1.1	101.34
600.00	602.23	2.94	0.49	100.37
750.00	744.21	27.78	3.73	99.23

Table 2. Precision and accuracy (analysis with spiked plasma samples at four different concentrations)

Spiked plasma concentration (ng/ml)	Within-run			Between-run		
	Concentration measured (*n*=6) (ng/ml) (mean ± S.D.)	CV (%)	Accuracy %	Concentration measured (*n*=30) (ng/ml) (mean ± S.D.)	CV (%)	Accuracy %
5.0	4.99±0.11	2.26	99.77	5.11±0.14	2.75	102.15
15.0	14.96±0.3	2.04	100.8	14.93±0.34	2.29	99.5
375.0	376.24±5.53	1.47	100.3	376.55±4.98	1.32	100.41
525.0	524.13±7.23	1.38	99.83	522.49±8.15	1.56	99.52

Table 3. Stability of the samples

Spiked plasma concentration (ng/ml)	Room temperature stability (26 h)		Autosampler sample stability (79 h)		Long term stability (76 days)		Freeze and thaw stability Cycle 3 (48 h)	
	Concentration measured (*n*=6) (ng/mL) (mean±S.D)	CV (%) (*n*=6)	Concentration measured (*n*=6) (ng/mL) (mean±S.D)	CV (%) (*n*=6)	Concentration measured (*n*=6) (ng/mL) (mean±S.D)	CV (%) (*n*=6)	Concentration measured (*n*=6) (ng/mL) (mean±S.D)	CV (%) (*n*=6)
15.0	14.65±0.17	1.16	16.03±0.31	1.92	14.97±0.51	3.38	14.47±0.19	1.32
525.0	516.20±10.63	2.06	558.67±3.67	0.66	528.83±5.78	1.09	524.15±9.07	1.73

Table 4. Pharmacokinetic data

Pharmacokinetic parameter	Values
C_{max} (ng/ mL)	27.96±11.52
AUC_{0-t} (ng.hr/mL)	296.47±12.5
$AUC_{0-\infty}$ (ng.hr/mL)	419.51±6.25
T_{max} (hr)	2.5
$T_{1/2}$	11.75±2.5
Kel	0.05897

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity
AUC_{0-t}: Area under the curve up to the last sampling time
C_{max}: The maximum plasma concentration
T_{max}: The time to reach peak concentration
Kel: Elimination rate

5. CONCLUSION

The proposed method was rapid, rugged and reproducible, higher in sensitivity than the previous reported method and the analyte was compared with analog based internal standard. The method described here was successfully applied to the pharmacokinetic study of EV healthy rats.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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