DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF SUVOREXANT IN RABBIT PLASMA BY HPLC-UV DETECTION

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Abstract: This work describes a specific and accurate high performance liquid chromatographic method for the determination of suvorexant in rabbit plasma using efavirenz as internal standard. Both suvorexant and the internal standard were eluted under isocratic mode using a Phenomenex C_{18} ODS 2, 250 X 4.6 mm i.d, 5 µm column. The run time of the method is 15.0 minutes. The elution was monitored at a wavelength of 245 nm. The extraction process involved a liquid-liquid extraction technique using methyl-t-butyl ether. The method showed good linearity in the range of 20.16-1008.00 ng/mL with a sensitivity (limit of detection) of 20.16 ng/mL using 300 µL of K_2EDTA plasma. The mean recovery of suvorexant from all the quality control samples is 62.99% with a coefficient of variation of 3.21% and recovery of internal standard was 25.62%. The intra-day accuracy at three levels of quality control samples ranged from 100.19 - 101.01% with a precision of 2.40 to 3.44%. The inter-day accuracy ranged from 99.97 – 101.75% with a precision of 2.37 – 4.07%. The peaks were well separated from the plasma interferences. The method is successfully validated as per FDA guidelines in human plasma containing K_2EDTA as an anti-coagulant.

***Keywords*:** Suvorexant; Insomnia; Neurokinin receptor; HPLC; MK-4305, Orexin Receptor.

I. INTRODUCTION

The usage of orexin receptor antagonists for treatment of insomnia is gaining importance over the recent years. Suvorexant was the first identified in 2007, [1] with equal affinity for OX_1R and OX_2R receptors. Suvorexant (MK-4305, ([(7R)-4-(5-chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan- 1-yl][5-methyl-2-(2H-1,2,3-triazol-2-yl)phenyl]methanone)) **[Fig-1a]** is similar in the mechanism of therapeutic action and has a diazepine based chemical structure. Suvorexant inhibits the wakefulness-promoting orexin neurons of the arousal system and promotes sleep [3–5]. The Food and Drug Administration has approved the usage of suvorexant in August 2014. Suvorexant must be administered at least 30 minutes prior to sleep. At a recommended daily dose is 10 mg, the onset of action is within 1 hour with a peak plasma concentrations (250–300 ng/mL) occurring within 2–3 h [8]. In humans, the drug is extensively protein bound (99%) and has a good bioavailability of 82%. [2, 3, 7]. Fecal elimination (66%) is the major route of elimination for Suvorexant while the urinary elimination is approximately 23% [3]. Suvorexant is commercially available as Belsomra®. The analysis of suvorexant by LC-MS/MS and GC-MS techniques in various biological fluids was reported earlier [6, 9]. We have developed the method and validated the method as per ICH Guidelines [10].

Our team is primarily focused on improving the oral bioavailability of suvorexant. Since suvorexant is a poorly soluble drug the absorption from the gastrointestinal tract is obviously a dissolution rate limited process. Our team had made several attempts to prepare formulations and subjected them to in vitro dissolution studies. After identifying few formulations with rapid dissolution (>90% within 5-10 minutes) it became imperative for us to test their absorption in pre-clinical subjects. Since the formulation design and optimization (either empirically or using QbD techniques) is tedious, we felt that the development of a HPLC-UV method is more rightful for preliminary estimations. While LC-MS/MS and GC-MS techniques offer more sensitivity, HPLC-UV methods are more versatile and offer the advantage of cost effectiveness. Accordingly we aimed to develop a suitable analytical method for the determination of suvorexant in rabbit plasma. The findings of formulation development are beyond the scope of this publication and therefore will be published later.

Analysis of Suvorexant in plasma samples by HPLC-UV detection is not reported till date elsewhere. In this paper, we described a simple liquid-liquid extraction technique for the determination of suvorexant using efavirenz [Fig-1b] as an internal standard. The sensitivity of the method is 20.16 ng/mL. The method is developed in rabbit plasma containing K2EDTA as the anti-coagulant.

II. MATERIALS AND METHODS

Solvents and Chemicals

Suvorexant reference standard was purchased from Beijing Mesochem Technology Co. Ltd., China. Efavirenz reference standard was gifted by M/s Aurobindo Pharma Limited, Hyderabad. Methyl tertiary butyl ether (HPLC grade) and Methanol (HPLC grade) were purchased from Merck Ltd, Mumbai. Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Acetone (GR grade) was purchased from SD Fine Chem Ltd, Mumbai. Dry Ice was procured locally. All other chemicals and reagents were of analytical grade.

Plasma Preparation

Rabbit plasma was purchased from National Institute of Nutrition (NIN), Hyderabad. The institutional ethical committee of NIN approved the plasma collection. Plasma was collected in our presence. Animals were handled carefully by well trained staff of NIN. Rabbits having average weight of 2.2 to 2.4 kg (Male & Female) were housed separately in standard cages as recommended by the institutional ethics committee and fed with standard diet. On the day of collection, blood was drawn from the marginal ear vein into vials containing K_2 EDTA as anticoagulant. The vials were allowed to stand for 10 minutes on the bench top and then centrifuged to separate the plasma. Plasma thus obtained was separated and pooled. Pooled plasma lots obtained on different days were separately labeled. These lots were stored below -20ºC freezer until use. Each pooled plasma was screened before use.

Chromatographic System

The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions software. The detector is set at a wavelength of 245 nm. Chromatographic separations were accomplished using a Agilent Zorbax 300º, (300 Extend – C_{18}), 5 µm, 150 mm×4.6 mm column. A mixture methanol and water in the ratio of 62.5 : 37.5 %v/v was used as the mobile phase. A mixture methanol and water in the ratio of 50 : 50 %v/v was used as the diluent solution. Both the solution mixtures were separately prepared and were filtered through 0.45 µm membrane (Millipore, Bedford, MA, USA) under vacuum, and then degassed by using a ultrasonicator. The mobile phase was pumped isocratically at a flow rate of 1.0 ml/min during analysis, at 24±2ºC temperature. The rinsing solution consists of a mixture of 60: 40 % v/v of Acetonitrile: HPLC Grade Water.

Preparation of Standard Solutions

A stock solution of suvorexant (1080 µg/ml) is prepared in methanol and labeled as master stock solution. The concentration is calculated on a dried basis taking into account for its potency and actual amount weighed. Stock solution of efavirenz (3000 µg/mL) is prepared methanol. This solution is labeled as Internal standard master stock solution. Both the solutions were stored below 10°C in a refrigerator. Stock dilutions of both drug and internal standard were prepared whenever required using diluent solution.

Sample Preparation

For the method development we have decided to construct a calibration curve having 6 non-zero standards. Aqueous stock dilutions in the range of 0.40 – 20.16µg/ml were prepared initially using diluent solution. 0.5 ml of each aqueous stock dilution is then transferred separately into individual labeled 10 mL volumetric flasks and made up to the mark using screened drug-free K_2EDTA rabbit plasma and mixed gently for 30 minutes using a rotating tumbler to obtain a homogenous plasma spiked solutions of desired concentrations. The final calibration standard concentrations are 0.0 (Blank; no suvorexant added), 20.16, 40.32, 201.60, 604.80, 806.40 and 1008.00 ng/mL. These calibration curve standards are labeled as CC-01 (LLOQ), CC-02, CC-03, CC-04, CC-05 and CC-06 (ULOQ) respectively. LLOQ is defined as the lower limit of quantification and ULOQ is defined as the upper limit of quantification for the calibration curve. Similarly quality control samples were prepared in plasma such that the final concentrations were 20.16, 60.48, 514.08 and 756.00ng/mL respectively. These samples are labeled as Lower limit of quantification (LLOQ QC), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively. Each of these spiked calibration standards and quality control samples were distributed in disposable polypropylene micro centrifuge tubes (2.0 mL, eppendorf) in volume of 0.5 mL and stored at -20°C until analysis.

Extraction procedure

The extraction of the plasma samples involved a simple liquid-liquid extraction technique. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 300 µL is then transferred to pre-labeled 2.0 mL polypropylene centrifuge tubes. 25 µL of internal standard (20 µg/mL of efavirenz in diluent solution) is then added and vortexed for thirty seconds. 1.0 mL of methyl tertiary butyl ether is then added mixed. The tubes were further vortexed for 5 min at 2200 rpm on a vibramax unit and then were centrifuged at 4000 rpm for 5 min in a refrigerated centrifuge at 4^0C. The vials were then allowed to flash freeze using a mixture of acetone and dry ice (approximate temperature of -80ºC). The supernatant is poured into pre labelled polypropylene tubes and allowed to evaporate to dryness under nitrogen at constant temperature of 40ºC for 10 minutes. The dried residue is then reconstituted in 100 µL of diluent solution, vortexed thoroughly and transferred into shell vials containing vial inserts for analysis. The injection volume is set at 20 µL for analysis. The autosampler temperature is maintained at 4ºC throughout the analysis. The column temperature oven is maintained at 24±2ºC.

Validation of quantitative HPLC method

The quantitative HPLC-UV method was validated to determine selectivity, calibration range, linearity, accuracy and precision, lower limit of quantification, % recovery, matrix effects and effect of short term, long term, freeze–thaw, auto sampler storage stability. The analysis is performed as per US FDA guidelines.

Selectivity

The selectivity of the method was evaluated by analyzing six independent drug-free K_2EDTA pooled plasma lots with reference to potential interferences from endogenous and environmental constituents.

Calibration curve

Calibration curves were generated in triplicate to confirm the relationship between the peak area ratios and the concentration of suvorexant in the standard samples. Fresh calibration standards were extracted and assayed. Calibration curves for suvorexant were represented by the plots of the peak-area ratio (suvorexant / efavirenz) versus the nominal concentration of the suvorexant in calibration standards. Suvorexant concentrations in QC samples, recovery, and stability samples were calculated from the resulting area ratio and the equation of the best fit line of the calibration curve.

Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and accuracies were determined by analyzing four QC levels on 3 separate days (n = 6 at each level) and the contents were estimated using the calibration curve of that corresponding day.

The accuracy of an analytical method describes how close the mean test results obtained by the method are to the nominal concentration of the analyte. Accuracy was calculated by the following equation, expressed as a percentage:

$$\text{Accuracy (\%)} = \text{mean observed concentration/nominal concentration} \times 100$$

The precision was expressed by co-efficient of variation (CV). The CV % indicates the variability around the mean in relation to the size of the mean, and is defined as:

$$\text{CV (\%)} = \text{standard deviation/mean observed concentration} \times 100$$

Stability Studies

Autosampler, and freeze–thaw stability of suvorexant was determined at low, medium and high QC concentrations. Bench top stability (6 hours), short term storage (7 days at -20ºC), long term storage stability (30 days in -20ºC) of the plasma matrix is also evaluated. For freeze-thaw stability, samples were allowed to undergo 3 freeze-thaw cycles (a freeze thaw cycle is defined as the removal of frozen plasma sample drawn from -20ºC freezer and thaw at Room temperature) with an intermittent duration of at least 12 hours between each cycle. The impact of freeze–thaw cycles on suvorexant concentration was studied. Following sample treatment/storage conditions, the suvorexant concentrations were analyzed in triplicates and compared to the control sample that had been stored at −20ºC. Autosampler stability of extracted samples was determined by comparing suvorexant concentration in freshly prepared samples and samples kept in auto sampler at 4ºC for 48 hrs. Aqueous solutions of suvorexant and internal standard were also evaluated for bench top stability (approx 6 hrs) and refrigerated stability for 30 days.

Recovery

Recovery was determined by comparing the area of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of suvorexant. This should highlight any loss in signal due to the extraction process. IS recovery was determined for a single concentration of 20 µg/mL.

Data analysis

HPLC data acquisition and processing was performed by Shimadzu LC Solutions Version 1.23 Version 1 software. Standard calibration curves for quantification of suvorexant were constructed by taking the peak-area ratio (suvorexant / efavirenz) versus the nominal concentration of the suvorexant. A best fit line is plotted and the equation of the best fit line is obtained. The accuracies of each of the calibration standards, quality control samples and stability samples were calculated using equation of the best fit line.

III. RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the determination of suvorexant in rabbit plasma. The authors have previously reported an analytical method based on HPLC-UV detection [10].

Suvorexant has a diazepine based chemical structure and the physicochemical properties were described elsewhere [10]. For estimation of suvorexant in plasma, we initially adopted the same chromatographic conditions of the analytical method reported earlier [10]. During our experiments, we found a lot of matrix effects that interfered with either the suvorexant and/or efavirenz peaks. Chromatographic separations were accomplished using a Agilent Zorbax 300^{o,} (300 Extend – C_{18}), 5 µm, 150 mm×4.6 mm column. A mixture methanol and water in the ratio of 62.5: 37.5 %v/v as the mobile phase at a flow rate of 1 ml/ min was best suitable for quantification. The detector is set at a wavelength of 245 nm.

Selection of Internal Standard

Lehrer [11] stated that a suitable internal standard should: 1) be completely resolved from all peaks in the sample, 2) be eluted near the analyte, 3) behave similarly to the analyte in pretreatment so that losses can be corrected, 4) have a peak area approximately equal to the standard in the concentration desired, 5) not normally be present in the sample, 6) be commercially available in a pure form, and 7) be easily added as a liquid. The search for an internal standard was carried out based on these principles. While preextraction and postextraction approaches are described [12-14], the preextraction method has the advantage of being able to compensate for sample loss during sample pretreatment [15]. The postextraction method, while it may not incur the same advantages as the preextraction method, is much easier to carry out because the optimization of the extraction process is only required for suvorexant instead of both suvorexant and the internal standard. Therefore, the simpler preextraction spiked internal standard method was selected. Furthermore, the advantage of a preextraction method will only materialize if the chosen internal standard behaves similarly to the analyte in pretreatment [11], which means that the recovery of suvorexant and the potential internal standard must be similar. However, in reality, the search for such an ideal internal standard can be difficult and time-consuming.

Among the various neutral drugs available with us, efavirenz emerged as the most reliable internal standard. Efavirenz had high lipophilicity and higher selectivity factor under the given column conditions. Therefore the late elution of Efavirenz did not result in interferences from plasma components. Under the aforesaid chromatographic conditions, Efavirenz eluted at 9.5 minutes while suvorexant eluted at 11.5 minutes. The column temperature oven is maintained at 24±2^{o}C.

Optimization of sample clean up

Initial experiments were performed using versatile methods such as liquid-liquid extraction (LLE), protein precipitation (PPT) and solid phase extraction techniques (SPE). Among these, PPT and LLE are relatively inexpensive as compared to SPE. Since LLE technique is known to give good sample clean-up and little or no noise (due to matrix components) [16], we evaluated LLE technique using solvents like diethyl ether, methyl-t-butyl ether, dichloromethane, n-hexane etc.

To identify the correct solvent for LLE process, plasma samples were initially spiked with known amount of the drug and homogenized. 300 µL of spiked plasma samples were equally distributed into separate vials. The pH of these spiked samples was then altered using various reagents (0.1 N HCl, 0.1 N NaOH, 0.1 $NaHCO_3$ etc). The effect of plasma pH on the recovery of suvorexant from spiked plasma samples was then studied using various solvents. Neutral pH (without any pretreatment of plasma) extraction using methyl-t-butyl ether resulted in acceptable recovery for suvorexant. An addition of 25 µL at a concentration of 20 µg/mL of internal standard resulted in best chromatography and is in accordance with the recommendations described earlier [11].

Detection and chromatography

Figure 3 shows the typical chromatograms of blank human plasma sample (A), a zero blank sample with Efavirenz (B), and (C) with a sample containing ULOQ sample extracted using internal standard indicating the specificity of the method. The retention times for suvorexant and internal standard were 9.50 minutes and 11.50 minutes, respectively.

IV. METHOD VALIDATION

Selectivity

The method was found to have high selectivity for the analytes; since no interfering peaks from endogenous compounds were observed at the retention time for suvorexant in any of the six independent blank plasma extracts evaluated (**Figure 3-A**).

Calibration curves

A system suitability exercise is performed before the initiation of the validation. A system is assumed to be suitable for analysis if and only if the % CV of 6 replicate injections for the retention times of suvorexant and internal standards is less

than 2 %. For preparation of pooled plasma for the validation, six different lots of blank pooled plasma lots were screened for specificity. All the lots were free of endogenous interferences at the retention times of the analyte and the internal standard. The results of specificity were demonstrated in **Table 1.**

Calibration curves for suvorexant were represented by the plots of the peak-area ratio (suvorexant / efavirenz) versus the nominal concentration of the suvorexant in calibration standards. The linear regression (represented as r2 values) was >0.99 for all curves generated during the validation. The calibration curve accuracy for plasma is presented in **Table 2a & Table 2b** demonstrating that measured concentration is within ± 15% of the actual concentration point (20% for the lowest point on the standard curve, the LLOQ). Results were calculated using peak area ratios. A representative calibration curve showing the regression equation and r2 value is depicted in **Figure – 2.**

Accuracy and precision

A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation is presented in **Table 3**. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was <5% for all QC concentrations, which was within the general assay acceptability criteria for QC samples according to FDA guidelines [17].

Lower limit of quantification

LLOQ has been accepted as the lowest points on the standard curve whose peak response of replicate injections has a coefficient of variation less than 20%. The LLOQ QC (sensitivity) was 20.16 ng/mL.

Carryover test

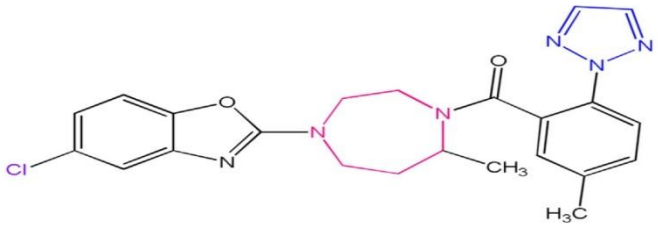
A critical issue with the analysis of many drugs is their tendency to get adsorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

Stability studies

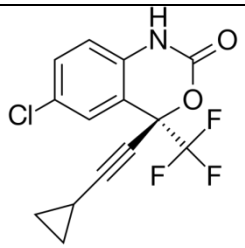
The results of bench top, long term, autosampler and freeze–thaw stability are presented in **Table 4a** and **Table 4b**. Suvorexant was found to be stable under all storage conditions. The stability of the aqueous solutions of suvorexant and the internal standard in neat solutions was also evaluated. Both drug and internal standard were found to be stable under the specified storage conditions.

Recovery

Recovery was determined by comparing the area of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of suvorexant. The mean recovery of suvorexant at LQC, MQC and HQC levels was 64.1 %, 66.3% and 58.6 % respectively. The overall recovery is 62.99 % with a % Coefficient of variation of 3.96%. The recovery of internal standard is 15.51 % at MQC level concentration of suvorexant.



A) Structure of Suvorexant



B) Structure of Efavirenz

Figure 1: Structure of Suvorexant and Efavirenz

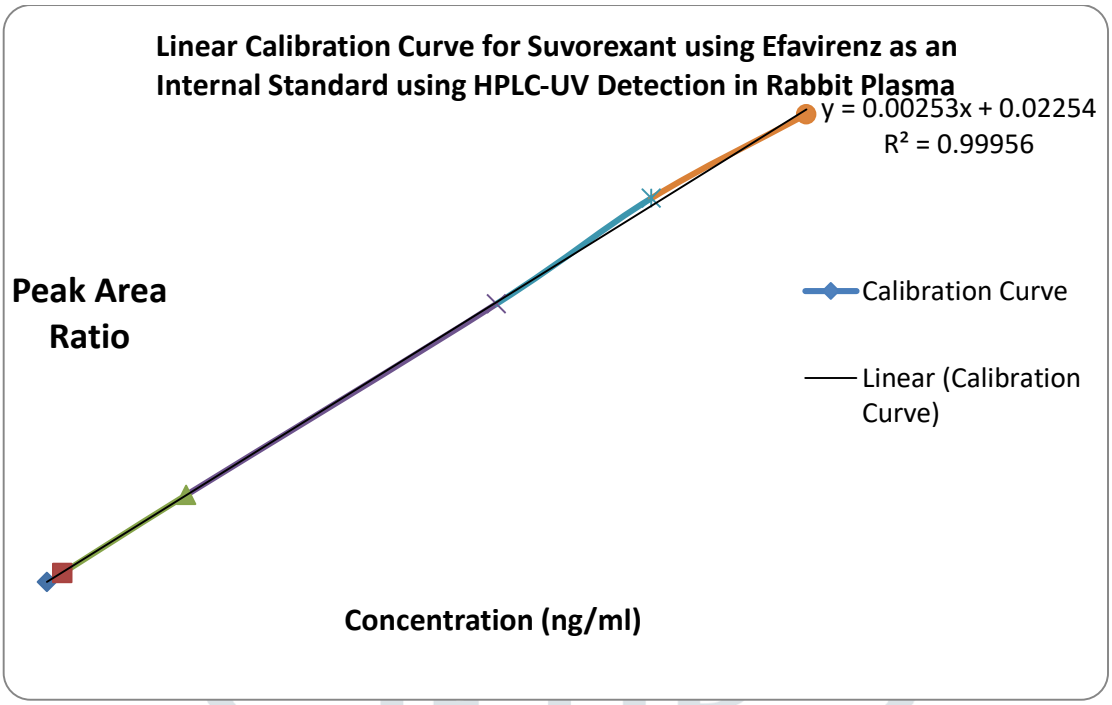


Figure 2: Calibration Curve of Suvorexant in rabbit plasma by HPLC-UV Detection.

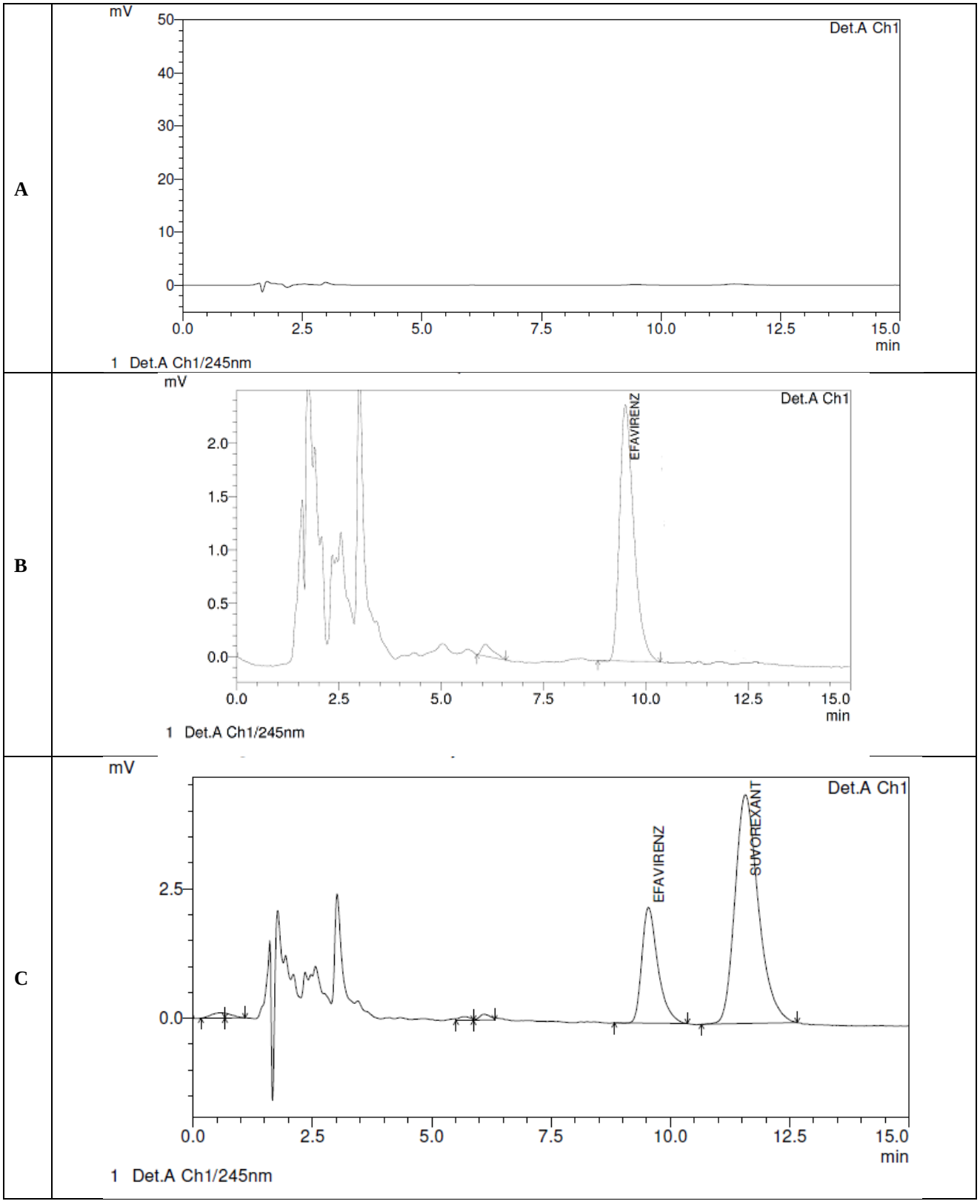


Figure 3: Chromatograms of (A) Extracted Blank Sample (B) Zero Blank Containing Efavirenz as Internal Standard (C) Suvorexant containing Efavirenz as Internal standard at ULOQ level.

Table 1: Specificity Exercise: Percent interferences at the retention times of the drug and the internal standards

Rabbit PLASMA ID	SUVOREXANT			INTERNAL STANDARD		
	RESPONSE IN BLANK	RESPONSE IN LLOQ	% INTER FERENCE	RESPONSE IN BLANK	RESPONSE IN LLOQ	% INTER FERENCE
1	137	4124	3.32	348	58592	0.59
2	112	4032	2.78	578	59473	0.97
3	192	4938	3.89	389	59387	0.66
4	193	4732	4.08	398	59837	0.67
5	187	4682	3.99	238	59372	0.40
6	183	4512	4.06	382	59383	0.64
Average	167.3	4503.3	3.686	388.8	59340.7	0.655
Total Number of lots	6	Number of lots meeting the requirements	6			
Percentage of Matrices meeting the selectivity criteria						100 %

Table 2a: Back calculated concentrations of Suvorexant and calibration curve parameters

CC-ID	CC-01	CC-02	CC-03	CC-04	CC-05	CC-06
Conc. (ng/mL)	20.16	40.32	201.60	604.80	806.40	1008.00
PA 01	19.59	38.71	202.16	601.19	812.50	997.65
PA 02	20.25	39.42	201.45	599.56	809.77	999.45
PA 03	20.81	40.11	200.56	603.32	809.46	1011.32
Mean	20.22	39.41	201.39	601.36	810.58	1002.81
±SD	0.61	0.70	0.80	1.89	1.67	7.43
%CV	3.03	1.77	0.40	0.31	0.21	0.74
%Nominal	100.28	97.75	99.90	99.43	100.52	99.48

Table 2b: Results of regression analysis of the linearity data

Linearity parameters	Mean (n = 3)
Slope	0.00259
Intercept	0.022533
Correlation coefficient (r^2)	0.99965

Table 3: Intra and Inter day accuracy and precision of HPLC assay

Parameters	Nominal Concentration (µg/mL)			
	HQC	MQC	LQC	LLOQ QC
	756.00	514.08	60.48	20.16
Precision and Accuracy Batch – 1				
Mean (n=6)	758.59	519.27	61.54	20.20
S.D.	18.173	17.858	1.495	0.556
% CV	2.40	3.44	2.43	2.75
Precision and Accuracy Batch – 2				
Mean (n=6)	762.485	521.983	61.873	20.298
S.D.	21.619	21.261	2.406	0.509
% CV	2.84	4.07	3.89	2.51
Precision and Accuracy Batch – 3				
Mean (n=6)	756.951	518.188	61.427	20.153
S.D.	17.973	19.257	2.303	0.506
% CV	2.37	3.72	3.75	2.51

Table 4a: Stability of suvorexant in plasma spiked samples under various conditions (n=6)

Storage condition	% Stability (LQC)	% Stability (HQC)
Bench top stability (6 Hours)	98.7	101.4
Short-term stability (7 Days at -20°C)	101.7	99.7
Long-term stability (30 Days at -20°C)	99.8	100.3
Freeze – Thaw stability (3 Cycles)	96.8	95.2
Auto sampler stability (4°C for 48 hours)	97.1	102.2%

Table 4b: Stability of suvorexant and internal standard in neat solutions (n=6)

Storage condition	% Stability of Suvorexant	% Stability of internal standard
Bench top stock solution stability (6 Hours)	101.3	99.3
Long-term stock solution stability (30 Days in refrigerator)	100.1	99.6
Bench top stock dilution stability (6 Hours)	98.2	99.1
Refrigerated stock dilution stability (72 hours in refrigerator)	99.7	99.4

V. CONCLUSION

Suvorexant estimation in various biological fluids was earlier reported using advanced techniques like LC-MS/MS or GC-MS. We developed a sensitive HPLC-UV based method that was not reported elsewhere. The matrix used is rabbit plasma and we have developed and validated as per US FDA guidelines. The extraction process was a simple LLE procedure using methyl-t-butyl ether. This assay requires only a small volume of plasma (300 µL). There is no carryover effect. Matrix effects are not observed. In conclusion, method validation following FDA guideline indicated that the developed method had high sensitivity with an LLOQ of approximately 20.16 ng/mL, acceptable recovery, stability, specificity and excellent efficiency with a total running time of 15.0 minutes per sample, which is significantly economical when compared to the cost of LC-MS/MS analysis. Thus this method can be suitable for pharmacokinetic studies of Suvorexant in rabbits.

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