CHAPTER 3

METHODS FOR THE ANALYSIS OF EFAVIRENZ

Drug profile and literature survey

3.1. Drug profile

Efavirenz is in a class of medications called non-nucleoside reverse transcriptase inhibitors (NNRTIS). It works by slowing the spread of HIV in the body. Efavirenz is a human immunodeficiency virus type 1 (HIV-1) specific, non-nucleoside reverse transcriptase inhibitor (NNRTI). It is never used alone and used in combination with other medicines to treat infection with human immunodeficiency virus type-1 (HIV-1), the virus that causes AIDS (Acquired immunodeficiency syndrome). Efavirenz activity is mediated predominantly by non-competitive inhibition of HIV-1 reverse transcriptase (RT). Efavirenz is also used with another medication to prevent infection in health care workers or other people who were accidentally exposed to HIV.

However, efavirenz does cause side effects, most notably ones affecting the central nervous system and involving symptoms such as dizziness, confusion, forgetfulness, abnormal dreams and difficulty in sleeping. These symptoms generally appear soon after treatment with efavirenz has started but in many patients, lessen or go away in the ensuing months.

Efavirenz

Efavirenz is chemically described as (S)-6-chloro-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one. Its empirical formula is C_{14}H_{9}ClF_{3}NO_{2}. Efavirenz (EFV) is a white to slightly pink crystalline powder with a molecular mass of 315.68 g/mol. It is practically insoluble in water. It has the following pharmacokinetic data: bioavailability 40-45 %, protein binding 99.5-99.75 %, half-life 40-55 hours. It has the following molecular structure:
Mode of action of efavirenz falls in the non-nucleoside reverse transcriptase inhibitor (NNRTI) class of antiretrovirals [1]. Both nucleoside and non-nucleoside RTIs inhibit the same target, the reverse transcriptase enzyme, and an essential viral enzyme, which transcribes viral RNA into DNA. Unlike nucleoside RTIs, which bind at the enzymes active site, NNRTIs bind within a pocket termed the NNRTI pocket. The usual adult dose of efavirenz is 600 mg per day (given at bedtime), or 800 mg daily when given concurrently with rifampicin as part of treatment of co-infection with tuberculosis.

**Duloxetine (internal standard)**

Duloxetine is chemically known as (+)-(S)-N-Methyl-3-(naphthalene-1-yloxy)-3-(thiophen-2-yl)propan-1-amine. Duloxetine has the molecular formula C_{18}H_{19}NOS and its molecular weight is 297.414 g/mol. Duloxetine is a white solid. It is slightly soluble in water. It has the following pharmacokinetic data: bioavailability ~ 50 %, protein binding ~ 95 %, and half-life 12.1 hours with excretion 70 % in urine, 20 % in faces. It has the following molecular structure;

![Molecular structure of Duloxetine](image)

The main uses of duloxetine are in major depressive disorder, general anxiety disorder, and stress urinary incontinence, painful peripheral neuropathy and fibromyalgia [2]. Duloxetine has demonstrated efficacy for the treatment of major depressive disorder. Recently, duloxetine was shown to be effective in elderly with recurrent major depressive disorder where it improved cognition, depression and some pain measures. The meta analysis of these trials indicated that the effective size of
duloxetine as compared with placebo was weak-to-moderate, and similar to other 11 antidepressants studied. The rationale behind the development of duloxetine was that inhibition of the reuptake of both serotonin and norepinephrine would make it work better than selective serotonin reuptake inhibitors (SSRIs), which inhibit only the reuptake of serotonin. However, in a comparative meta analysis of clinical trials duloxetine appeared to be insignificantly less effective than SSRIs. A head-to-head comparison of duloxetine with a SSRIs escitalopram (Lexapro) found duloxetine to be both less tolerable and less effective.

3.2. Literature review

Dogan-Topal et al. [3] have developed a reverse phase high performance liquid chromatographic method with diode array detection procedure for the simultaneous determination of abacavir, efavirenz and valganciclovir in spiked human serum. Separation was performed on a Waters spherisorb column with acetonitrile: methanol: KH$_2$PO$_4$ (pH-5.0) (40:20:40v/v/v) isocratic elution at a flow rate of 1.0 mL/min. Calibration curves were constructed in the range of 50 - 30,000 ng/mL for abacavir and efavirenz, and 10- 30,000 ng/mL for valganciclovir in serum samples. The limit of detection and limit of quantification were 3.80 and 12.68 ng/mL for abacavir, 2.61 and 8.69 ng/mL efavirenz, and 1.30 and 4.32 ng/mL for valganciclovir.

Sailaja et al. [4] have reported a liquid chromatographic method for the analysis of efavirenz in human plasma, chromatography was performed with C$_{18}$ analytical column and 50:50 acetonitrile-phosphate buffer (pH 3.5) was used as mobile phase. Compounds were monitored by uv-detection at 247 nm. The retention time for efavirenz was 6.45 min and that of internal standard nelfinavir was 2.04 min. Response was a linear over the concentration range of 0.1 - 10 µg/mL in human plasma.

A high performance liquid chromatography/positive ion electrospray method for the simultaneous quantification of efavirenz, emtricitabine and tenafavir was developed and validated with 100 µL human plasma following solid phase extraction by Nirogi et al. [5]. Analytes were separated using a gradient mobile phase on a reverse phase column and analyzed by MS/MS in the multiple reaction monitoring mode. Response was a linear over the concentration range of 20-2000 ng/mL, 2-200
ng/mL and 20-2000 ng/mL in human plasma for efavirenz, emtricitabine and tenofovir, respectively.

Sarasa-Nacenta et al. [6] have established a HPLC method with ultraviolet detection in human plasma. The method involved solid phase extraction of the drug and the internal standard (L-737, 354) from 300 µL of human plasma. The analysis was via uv-detection at 250 nm using a reversed phase C₈ column and an isocratic mobile phase consisting of phosphate buffer (pH-5.75) and acetonitrile in the ratio 55:45 (v/v).

Veldkamp et al. [7] have described HPLC method for the quantification of efavirenz in human plasma suitable for therapeutic drug monitoring in plasma. Sample pretreatment consists of protein precipitation with acetonitrile and the drug is separated by isocratic reversed phase high performance liquid chromatography with uv-detection at 246 nm. The method has been validated over the range of 10-10000 ng/mL using a volume of 250 µL plasma.

Viana et al. [8] explained a simple assay method by HPLC for efavirenz in tablet. Analyses were performed by uv detector at 252 nm, on a reverse phase column C18, 250 mm x 3.9 mm, 10 µm using isocratic mobile phase containing acetonitrile/water/orthophosphoric acid (70:30:0.1).

Mogatle and Kanfer [9] have described a pharmacokinetic interaction study of efavirenz (EFV), mobile phase consisting of 0.1 M formic acid, acetonitrile and methanol (43:52:5) and it was pumped at a low flow rate of 0.3 mL/min through a reverse phase Phenomenex® Luna C18 (2) (5 µm, 150 mm × 2.0 mm i.d.) column maintained at 40 °C. Diclofenac sodium was used as an internal standard (IS) and EFV and IS were monitored at 247 and 275 nm, respectively. Sample preparation involved the addition of mobile phase to 100 µL of plasma to precipitate plasma proteins followed by direct injection of 10 µL of supernatant onto the column.

In another method [10], the determination of efavirenz from human plasma using high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) was developed. The incurred sample reanalysis was performed and the data of incurred sample reanalysis was subjected to two different statistical approaches. The incurred sample reanalysis program was carefully designed to provide additional
data to improve confidence in reliability and reproducibility of the validated method. The analytical method involved solid phase extraction of efavirenz using Waters oasis HLB 1cc/30 mg cartridges. The samples were chromatographed on Hypurity advance, 50 x 4.6 mm, 5 μm column using a mobile phase consisting of 5 mM ammonium acetate solution:acetonitrile (10:90 v/v).

Hamrapurkar et al. [11] have reported a stability-indicating high performance liquid chromatographic (HPLC) method for analysis of efavirenz in the presence of the degradation products generated in the stress degradation study. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal decomposition. Extensive degradation was found to occur in alkaline medium and under thermal stress. Minimum degradation was observed under acidic medium, in the photolytic conditions and oxidative stress. Separation of drug from degradation products formed under stress conditions was achieved on a C-8 column using acetonitrile:potassium dihydrogen phosphate (pH 2.9, 25 mM) - (60:40, v/v) as the mobile phase. The flow rate was 1 mL/min and the detector was set at in a range of wavelength between 220 nm to 390 nm.

Potale et al. [12] have described a stability indicating high-performance thin layer chromatographic method for analysis of efavirenz. The method employed TLC aluminium plate’s pre coated with silica gel 60 F 254 as the stationary phase. The solvent system consisted of dichloromethane:methanol (5:0.3, v/v). This system was found to give compact spot for efavirenz (Rf value 0.72 ± 0.03). Efavirenz was subjected to stress test conditions like acid, alkali, neutral hydrolysis, oxidation, and dry heat and photo degradation. The spot for product of degradation were well resolved from the drug. Densitometric analysis of drug was carried out in the absorbance mode at 247 nm. The linear regression data for the calibration plots showed good linear relationship with r² was 0.998 in the concentration range of 400-2000 ng/spot.

A RP-HPLC method [13] was described for the determination of lamivudine, tenofovir and efavirenz simultaneously in combined tablet dosage form. The mobile phase used was a mixture of phosphate buffer pH 4 and acetonitrile (42:58 % v/v). UV detector at 254 nm carried out the detection of lamivudine, tenofovir and efavirenz. The retention time of lamivudine, tenofovir and efavirenz were found to be
2.220, 3.276 and 10.814 min respectively. The linearity of the method was studied in 25 % to 150 % targeted concentration and regression coefficient for all three drugs was found to be 0.999.

Reverse phase high performance liquid chromatography methods have been developed for the simultaneous estimation of efavirenz, lamivudine and zidovudine in tablet dosage form [14]. In reverse phase high performance liquid chromatography analysis is carried out using acetonitrile, methanol and 0.05 M di potassium hydrogen orthophosphate in the ratio of 40:40:20 (v/v/v) as the mobile phase and Luna C18 (4.6 x 250 mm) column as stationary phase with detection wavelength of 259 nm. Linearity was obtained in the concentration range of 100 - 200, 15 - 45 and 40 - 120 μg/mL for efavirenz, lamivudine and zidovudine, respectively.

An isocratic HPLC method for the assay of efavirenz (EFA) in bulk and pharmaceutical dosage forms was reported [15]. The chromatographic conditions comprise of a Novapak phenyl column. A mixture of phosphate buffer and acetonitrile was used as mobile phase. Quantitation was achieved by uv-detection at 247 nm. A linear response ($r^2 = 0.999$) was observed in the range of 0.05-0.15 mg/mL.

Pradeep Kumar et al. [16] have developed high performance thin layer chromatographic method for the estimation of efavirenz in tablet dosage forms. The method employed TLC aluminium plate’s pre coated with silica gel 60 F 254 as the stationary phase. The mobile phase used was a mixture of toluene:ethyl acetate:formic acid (10:3:1, v/v). The detection of spot was carried out at 254 nm. The calibration curve was found to be linear in the range 300-1800 ng/mL with regression coefficient of 0.9991.

Identification and characterization of efavirenz metabolites were reported by Mutlib and coworkers [17]. To examine the potential differences in the metabolism among species, liquid chromatography/mass spectrometry profiles of efavirenz metabolites in urine of rats, guinea pigs, hamsters, cynomolgus monkeys, and humans were obtained and compared. The metabolites of efavirenz were isolated, and structures were determined unequivocally by mass spectral and NMR analyses. The biotransformation pathways of efavirenz in different species were proposed based on some of the in vitro results.
Kwon-Bok Kim and co-workers [18] have developed a method for determining efavirenz, 8-hydroxyefavirenz, and 8,14-dihydroxyefavirenz in human plasma simultaneously using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Three compounds and ritonavir, an internal standard, were extracted from plasma using ethyl acetate in the presence of 0.1 M sodium carbonate after incubation of β-glucuronidase (500 U). After drying the organic layer, the residue was reconstituted in mobile phase (acetonitrile: 20 mM ammonium acetate, 90:10 v/v) and injected onto a reversed-phase C18 column. The isocratic mobile phase was eluted at 0.2 mL/min. The retention time is 1.93, 1.70, 1.52, and 1.82 min for efavirenz, 8-hydroxyefavirenz, 8,14-dihydroxyefavirenz, and ritonavir, respectively.

A simple RP-HPLC method for simultaneous estimation of emtricitabine, tenofovir and efavirenz was described in the literature [19]. Chromatography was carried on a column X-terra RP-18 using gradient composition of ammonium acetate buffer as mobile phase A and acetonitrile as mobile phase B at a flow rate of 1.0 mL/min, detection at 260 nm. The retention times of the emtricitabine, tenofovir disoproxilfumarate and efavirenz was about 4.61, 7.52, 9.10 min, respectively. The linearity was found to be in the range of 50 - 150 μg/mL for emtricitabine, tenofovir and efavirenz.

A reverse phase-high performance liquid chromatographic method for the simultaneous determination of lamivudine and efavirenz in tablet dosage form is developed and validated [20]. The chromatographic analysis was performed on a Thermo BDS hypersil C 18 column (250×4.6 mm, 5 μm) in isocratic mode, the mobile phase consisted of methanol, acetonitrile and 0.05 M phosphate buffer (adjusted to pH 4.5 with ortho-phosphoric acid) at a ratio of 60:20:20 (v/v/v), and a flow rate of 1 mL/min. The eluents were monitored at 254 nm. The retention time of lamivudine and efavirenz were found to be 2.50 and 4.25 min, respectively. The linear ranges were found to be 10 – 60.22 μg/mL for lamivudine and 10-60 μg/mL for efavirenz.

A high performance liquid chromatographic method was employed for quantitative determination of efavirenz, lamivudine and tenofovir disoproxil fumarate in active pharmaceutical ingredients and their dosage forms [21]. The method is applicable to the quantification of related compounds of efavirenz, lamivudine and
tenofovir disoproxil fumarate from one of the fixed dosage combinations. Chromatographic separation of drugs from the possible impurities and the degradation products was achieved on an ACE C18, 250 x 4.6 mm, 5.0 μm column; the gradient elution achieved within 120 min. Dilute ammonium acetate was used as mobile phase A, and degassed mixture of acetonitrile and methanol (40:60) was treated as mobile phase B. The flow rate was 1.5 mL/min, and the detection was done at 265 nm. The above developed HPLC method was further subjected to hydrolytic, oxidative, photolytic and thermal stress conditions.

Bedor et al. [22] have developed a LC-MS/MS method for the estimation of efavirenz with hydrochlorothiazide used as an internal standard. Chromatographic separation was performed on an Onix C18 column (50 x 4.6 mm I.D., monolithic). Isocratic elution mode of the analytes from the column was achieved with a mobile phase consisting of acetonitrile/water (50:50 v/v+5% of isopropyl alcohol) at a flow rate of 1.5 mL/min. The linear concentration range was 100-5000 ng/mL.

A validated isocratic HPLC method [23] was utilized for the assay of efavirenz. The method employs a Zodiac C18 column with methanol and acetonitrile (80:20 v/v) as the mobile phase and uv detection at 280 nm. A linear response (r > 0.998) was observed over the concentration range of 15-45 μg/mL.

Theron et al. [24] have explained LC-MS/MS method for the analysis of EFV in biological fluids. Sample preparation of the saliva involved solid phase extraction (SPE) on C18 cartridges. The analytes were separated by high performance size and detected with tandem mass spectrometry in electrospray positive ionization mode with multiple reaction monitoring. Gradient elution with increasing methanol concentration was used to elute the analytes, at a flow rate of 0.4 mL/min. The total run time was 8.4 min and the retention times for the internal standard (reserpine) was 5.4 min and for EFV was 6.5 min. The calibration curves showed linearity over the concentration range of 3.125–100 g/L.

Madesh et al. [25] have described an isocratic reversed-phase HPLC method for the simultaneous determination of lamivudine (LAM), tenofovir (TEN) and efavirenz (EFV) in tablets. The chromatographic separation was carried out on BEH symmetry C18 (50 x 4.6 mm, 1.7 μm) column with a mixture of methanol: phosphate
buffer pH 3.0 (65:35, v/v) as mobile phase; at a flow rate of 0.3 mL/min. The retention times for LAM, TEN and EFV were observed to be 0.432, 0.657, 2.281 min, respectively. Calibration range was linear over the concentration range of 10-50 μg/mL for LAM and TEN; and 20-100 μg/mL for EFV.

A high performance liquid chromatography was developed and validated for efavirenz in tablets by Osnir de Sá Viana et al. [26]. The physical chemical characteristics of efavirenz were investigated to developing the method. Analyses were performed by an ultraviolet detector at 252 nm wavelength, on a reverse-phase column (C18, 250 mm x 3.9 mm, 10 μm), using an isocratic mobile phase containing acetonitrile /water/ ortho phosphoric acid (70:30:0.1, v/v/v).

Monic Inbaraj [27] has developed a LC-MS/MS method for efavirenz using nevirapine as an internal standard. The analyte extracted from solid phase extraction (SPE). Chromatographic separation was achieved from RP C18. The linear range of this method was 50-5000 ng/mL.

Huang et al. [28] have reported an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for quantification of commonly used antiretroviral drugs ritonavir (RTV), indinavir (IDV), atazanavir (ATV), and efavirenz (EFV) in mouse serum and tissues (liver, kidney, lung, and spleen). Chromatographic separation was achieved using a gradient mobile phase (5 % acetonitrile in methanol and 7.5 mM ammonium acetate (pH 4.0)) on an ACQUITY UPLC (®)BEH Shield RP 18 column. All compounds eluted within a 7 min run time. Lopinavir was used as an internal standard. Detection was achieved by dual positive and negative ionization modes on a quadrupole linear ion trap hybrid mass spectrometer with an electrospray ionization (ESI) source. The dynamic range was 0.2-1000 ng/mL for RTV, IDV, and ATV, and 0.5-1000 μg/mL for EFV.

Avery and coworkers [29] developed an UPLC/MS-MS method for quantitation of protein free efavirenz (EFV) as well as total concentrations of EFV in human blood and seminal plasma. The analytical method possesses capabilities for concentration measurements of EFV ranging from 0.5 - 10,000 ng/mL. The method employs a racemic fluorinated analog of EFV (F-EFV) as the internal standard. EFV and F-EFV were eluted from a reverse-phase UPLC column via gradient elution with
detection via negative ion multiple reaction monitoring (MRM). EFV and F-EFV were detected via the following MRM transitions: m/z 314.0>244.1 and m/z 298.0>227.9. The time required for the analysis of each sample was 8.0 min.

Apart from chromatographic methods, few spectrophotometric methods have been proposed for EFV determination. The author has given literature survey on these methods in the following paragraphs.

Suhas et al. [30] have reported a ultra-violet spectrophotometric method for the estimation of efavirenz (EFA) in pure drug and in pharmaceutical formulation. Linear response obtained was in the concentration range of 5 - 40 μg/mL with correlation coefficient of 0.9993 and 0.9989 in solvent and urine, respectively.

Kumaraswamy and coworkers [31] have described a uv - spectrophotometric method for the analysis of efavirenz in blend and in tablets formulations. The method was based on simple uv estimation in cost effective manner for regular laboratory analysis. The instrument used was Perkin Elmer, uv spectrophotometer and using 0.1 N NaOH as solvent system. Sample was analyzed using UV Win Lab 5.2.0 Software and matched quartz cells 1 cm and was monitored at 302 nm. Linearity was obtained in the concentration range of 2 - 10 mg/mL for efavirenz.

Vishnu Vardan et al. [32] have described the development of an uv-spectrophotometric method for the simultaneous determination of efavirenz, lamivudine and tenofovir disoproxil fumarate. The absorption maxima of the drugs were found to be 247, 273 and 258 nm for efavirenz, lamivudine and tenofovir disoproxil fumarate respectively in acetonitrile: water (50:50, v/v) solvent system. Linearity was observed over a range of 1-20 μg/mL for efavirenz, 1-10 μg/mL for lamivudine and tenofovir disoproxil fumarate.

In another spectrophotometric method [33], a quantitative estimation of efavirenz in bulk and tablets was described, in methanol: water (80:20, v/v), efavirenz exhibits an absorption maximum at 245 nm and method obeys Beer’s law at the concentration range of 5-50 μg/mL.

Arnaldo et al. [34] developed a stripping voltammetry method for the determination of the antiretroviral drug of efavirenz at the sub micro molar
concentration levels in diluted alkaline electrolyte. Optimum experimental conditions were: $2.0 \times 10^{-3}$ mol/L NaOH, accumulation potential of $-0.10$ V, pulse amplitude of 50 mV and scan rate of 50 mV/s. The response is linear over the concentration range of 0.01-0.25 ppm. For an accumulation time of 10 min, the limit of detection was 1.0 ppb ($3.0 \times 10^{-9}$ mol /L).

A new kind of method; the thermal decomposition of efavirenz (non-nucleoside reverse transcriptase inhibitor with a prolonged half-life) studied using differential scanning calorimetry (DSC) and thermo gravimetry/derivative thermo gravimetry (TG/DTG) by Viana et al. [35]. Non-isothermal method was employed to determine kinetic data of decomposition process. The physical chemical properties and compatibilities of several commonly used pharmaceutical excipients (Methocel®K100, magnesium stearate, crospovidone, croscarmelose, microcrystalline cellulose 101) with efavirenz (EFV) were evaluated using thermo analytical methods. The thermal kinetic TG analysis under nitrogen atmosphere was studied at the heating rate of 5, 7, 5, 10, 15 and 20 ºC min$^{-1}$. The activation energy ($E_a$) and the pre-exponential factor (log $Z$) were obtained by means Flynn-Ozawa-Wall (FWO) and Ozawa methods.

None of the HPLC or LC-MS/MS methods reported for the analysis of efavirenz in CPDA plasma. Several reported methods were used mouse serum, saliva, and tissue for its analysis. The author has developed cost effective methods which would overcome the shortcomings of the existing methods. In the present investigation, the author has proposed LC-MS/MS and HPLC methods for EFV analysis in CPDA plasma. The details about the method development and validation are presented in Section 3A (LC-MS/MS) and Section 3B (HPLC).
Section-3A

Quantitative determination of efavirenz in human CPDA plasma by sensitive liquid chromatography, tandem mass spectrometry

3A.1. Introduction

CPDA an anticoagulant solution, containing citric acid, sodium citrate, monobasic sodium phosphate, dextrose, and adenine. It was used for the preservation of whole blood and red blood cells for up to 35 days; it extends red cell survival by providing adenine needed for the maintenance of red cell ATP levels. The official USP name for CPDA anticoagulant is citrate phosphate dextrose adenine solution.

3A.2. Experimental

3A.2.1. Materials

Efavirenz drug substance was obtained from Wockhardt Research Center (Aurangabad, India). Duloxetine (IS) was obtained from Cipla Ltd, Bangalore. n-hexane was obtained from Merck Mumbai and ethyl acetate was purchased from Qualigens fine chemicals, Mumbai. Acetonitrile and methanol were purchased from Merck, Mumbai. Ammonia solution (purity 28%) was purchased from Merck, Mumbai.

3A.2.2. Reagents

Ethylacetate: n-hexane (60:40, v/v): It was prepared by mixing 300 mL of ethyl acetate and 200 mL of n-hexane in a reagent bottle and mixed well. The same was used for analysis.

0.2% formic acid: It was prepared by transferring 2 mL of formic acid into a 1000 mL volumetric flask containing milli-Q water and mixed well.

Mobile phase (0.2% formic acid: acetonitrile, 20:80, v/v): 200 mL of 0.2% formic acid and 800 mL of acetonitrile were transferred into a 1000 mL reagent bottle and mixed well. It was filtered through 0.45 μm filter paper and degassed by sonication before use.
**NH₃ solution:** It was prepared by mixing 1 mL of ammonia solution (28% purity) in to 100 mL volumetric flask containing milli-Q water mixed well before use.

**Methanol: milli-Q water (1:1, v/v) (diluents):** A volume of 100 mL each of the water and methanol was transferred into a reagent bottle using a measuring cylinder and mixed well. This solution was used as a diluent.

**3A.2.3. Operating conditions and equipments**

The high performance liquid chromatograph SIL HTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-AD VP binary pump, a DGU20A5 degasser, and a SIL-HTC auto sampler equipped with a CTO-10AVP thermostated column. The chromatograph has Zorbax_SB_C8 column (5 µm, 100 mm x 4.6 mm id) and column oven temperature was maintained at 30 °C. Isocratic mobile phase flow was performed throughout the run. It was pumped at a flow rate of 0.8 mL/min with an injection volume of 10 µL.

Mass spectrophotometer detection was performed on an API-3000 triple quadrupole instrument (AB SCIEX, Toronto, Canada) using the multiple reaction-monitoring (MRM) modes. Turbo electro spray ionization (ESI) interface in positive mode was used. The main working parameter of the mass spectrometer was summarized in **Table 3A.1.** Data processing was performed on analyst 1.4.1 software.

**Table 3A.1. Main working parameters of the tandem mass spectrometer**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source temperature</td>
<td>375 °C</td>
</tr>
<tr>
<td>Dwell time/transition</td>
<td>200 msec</td>
</tr>
<tr>
<td>Curtain gas</td>
<td>12 psi</td>
</tr>
<tr>
<td>Ion spray voltage</td>
<td>5500 V</td>
</tr>
<tr>
<td>Entrance potential</td>
<td>4 V (analyte). 8(IS)</td>
</tr>
<tr>
<td>Declustering potential (DP)</td>
<td>35 V</td>
</tr>
<tr>
<td>Collision energy (CE)</td>
<td>9.5 eV (analyte).19 (IS)</td>
</tr>
<tr>
<td>Collision cell exit potential (V) CXP</td>
<td>9.5 eV (analyte).15 (IS)</td>
</tr>
</tbody>
</table>
Focusing potential 99 eV (analyte.170 (IS)
Nebulizer 8.0 psi
CAD gas 10 psi
Ion transition for duloxetin Q1→298.4, Q3→154.10
Ion transition for efavirenz Q1→316.10, Q3→244.00

3A.2.4 Preparation of standards samples and calibration curves

Stock solutions of efavirenz (1.0 mg/mL) and IS (1.0 mg/mL) were prepared in methanol. All stock solutions were stored at 2-8 °C. Working standard solutions of the analyte were prepared by further dilution of the stock solution with methanol:water::1:1(v/v) to give a series of concentrations(1.54, 3.07, 10.97, 21.94, 54.85, 109.69, 182.82, 228.53 µg/mL). IS working solution was prepared by further dilution of the stock with methanol:water (1:1 v/v) to give the concentration of 30 ng/mL. The calibration samples were prepared by spiking 100 µL of corresponding working standard solutions of the analyte into 4.90 mL of blank human plasma to give a calibration range of 0.03–4.57 µg/mL (0.03, 0.06, 0.22, 0.44, 1.10, 2.19, 3.66 and 4.57µg/mL.). The samples were then subjected to the sample processing procedure described in Section 3A.2.5. Calibration curve was obtained by plotting the peak area ratios of efavirenz to IS against the nominal concentrations of calibration standard samples, the amount of drug was computed from the standard calibration graph [Fig. 3A.1] or regression equation. Samples at three levels of concentrations [0.03 (LLOQ), 0.08 (low), 1.4 (medium), and 3.5 µg/mL (high)] for method validation and quality control were prepared in the same way.
Fig. 3A.1 Calibration graph of efavirenz (µg/mL)

01_05_09_EFA_PA4.rdb (EFAVIRENZ): "Linear" Regression ("1 / (x * x)" weighting): $y = 1.11x - 0.00385$ ($r = 0.9993$)
3A.2.5. Sample preparation procedure

A 100 µL aliquot of plasma sample was added to 50 µL aliquot of IS except blank. The samples were basified with 100 µL of NH₃ solution in water. Then, samples were extracted from 2.5 mL of extraction mixture (ethyl acetate: n-hexane, 60:40 v/v), the samples were mixed in a vibramax for 10 min, and centrifuged at 4500 rpm for 5 minutes. The organic layer (2.0 mL) was transferred into a series of test tubes, and evaporated to dryness under stream of nitrogen at 40 °C about 15 min. The residue was reconstituted in 0.5 mL of mobile phase followed by vortex, and then inject 10 µL of sample on to system. Data processed with analyst software 1.4.1.

3A.3. Bio-analytical method validation

The method was validated for selectivity, linearity, accuracy, precision, extraction recovery and stability according to the FDA guidelines [36] for validation of bioanalytical methods. Validation runs were conducted on three consecutive days. Each validation run was consisted of two sets of calibration standards and six replicates of QC plasma samples at three concentrations. The peak area ratios of efavirenz to I.S. of QC samples were interpolated from the calibration curve on the same day to give the concentrations of efavirenz. The results from QC plasma samples in three runs were used to evaluate the precision and accuracy of the method developed. The selectivity of this method was investigated by preparing and analyzing six individual human blank plasma samples with corresponding plasma samples spiked with efavirenz (LLOQ) and duloxetine (IS). The linearity was determined in the range of 0.03–4.57 µg/mL by plotting the peak area ratio of efavirenz to I.S versus the nominal concentration of efavirenz in plasma. The calibration curves were constructed by weighted (1/x²) least squares linear regression. The precision and accuracy were determined by repeated analysis of six replicates at each QC level (LLOQ, low, mid and high levels) on three different days. The precision was defined as relative standard deviation (R.S.D.) and the accuracy was expressed as relative error (R.E.). The extraction recovery of efavirenz at three QC concentrations was determined by comparing the peak area of extracted samples spiked with known amount of the analytes with that of spiked post-extraction at corresponding concentrations. Stability experiments were performed to evaluate the stability of
efavirenz in plasma samples under different temperature and timing conditions. Five aliquots of QC plasma samples of low and high concentrations were subjected to the following conditions: four freeze thaw (room temperature) cycles, storage at −50 °C for 30 days. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 9 h that exceeded the routine preparation time of samples. Post-preparative stability was assessed by analyzing the extracted QC samples kept in the auto sampler at 5 °C for 48 h.

3. A.4. Results and discussion

3. A.4.1. Mass spectrometry optimization

The mass spectrometer was tuned in both positive and negative ESI modes for both efavirenz and duloxetine. Signal intensity obtained in positive mode was much greater than those in negative ionization mode, the analyte and internal standard formed predominantly protonated molecule [M+H]+, no adduct ions were detected. Efavirenz gave an intense product ion at m/z Q3→244.00, Q1→316.00. And IS duloxetine gave an intense product ion at m/z Q3→154.10, Q1→298.40. The collisionally activated dissociation (CAD) mass spectrum of efavirenz shows the formation of characteristic product ions at m/z 244.00 and the CAD mass spectrum of the IS shows the formation of characteristic product ions at m/z 154.10. The most sensitive mass transition m/z 316 to 244 for efavirenz and m/z 298.4 to 154.1 for IS was observed. The parameters such as source temperature, declustering potential, focusing potential and flow rate of gases were optimized to obtain highest intensity of protonated molecules of the efavirenz and duloxetine.

The analysis of efavirenz in human plasma is of major interest in pharmaceutical research, pharmacokinetic applications, requiring highly selective assays with high sample through output capacity. Quantification of drugs in biological matrices by LC-MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique in recent years. The ESI was chosen since the sensitivity and linearity for the analyte were better by positive ESI mode LC/MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus the MRM technique was chosen for the assay developments. The MRM state file parameters were optimized to
maximize the response for the analyte. The parameters presented in the Table 3A.1 are the results of this optimization.

3. A.4.2. LC conditions and optimization

Several chromatographic trials were taken to achieve the good chromatographic, resolution and symmetric peak shapes for the analyte and IS as well as a short run time. Optimization of mobile phase is important for improving peak shape, detection sensitivity and shortening run time of efavirenz and duloxetine. Methanol and acetonitrile were both attempted as the organic modifier of mobile phase. It was found that peak shape was more symmetric in acetonitrile compared to methanol; even in lower concentration peak shape was good. Response of analyte was crucial for quantification, therefore acetonitrile was chosen as organic phase. Addition of formic acid in mobile phase was increased the ionization of efavirenz and duloxetine. The concentration of formic acid was also checked between 0.1 % and 1 % for both analyte and IS which were found to have highest response in the mobile phase with 0.2% formic acid. Finally, the mobile phase of composition 80% organic solvent and 20% of 0.2% formic acid in water was fixed. IS retention time 1.6 min and analyte retention time 2.8 min were obtained. A flow rate of 0.8 mL/min produced with good peak shape and total run time for a sample is 3.5 min. Column trials were also taken with different types of columns, in BDS hypersil C18 column split peak was observed but response was good whereas in Hypersil gold column, peak found at high run time of efavirenz of 9.2 min, Inertsil ODS 3V column response was less and LLOQ peak shape also not good, while in Discovery C8 column no retention means in 0.4 min efavirenz was eluted. So, finally good resolution and peak shape were observed in Zorbax_SB_C8 5 cm column. Thus, it was used throughout the experiment for the analysis of EFV in human plasma.

3. A.4.3. Sample preparation procedure

In respect to the high sensitivity requirement for this assay, a protein precipitation method was excluded because of dilution of the sample after sample preparation. Most published methods were utilized SPE extraction methods followed by reconstitution to a small volume. The overall recovery was always below 80% and sample blockage in the LC system. The problem identified was in the step of sample
elution, sample preparation method was switched to liquid-liquid extraction. Several conditions were evaluated: n-hexane-ethyl acetate (8:2), methyl t-butyl ether, and diethyl ether. However, SPE is more costlier than liquid-liquid extraction technique, to minimize the cost, in liquid-liquid extraction was utilized to optimize the preparation.

Finally, liquid-liquid extraction (LLE) was used for the sample preparation in this work. LLE gives spectroscopically cleaned samples and avoiding the introduction of non volatile materials on to the column and MS systems. For LC-MS/MS analysis, cleaned samples are essential for minimizing ion suppression and matrix effect. A mixture of ethyl acetate: n-hexane in ratio 60:40 v/v was found to be optimal. This can produce a neat chromatogram for a blank plasma sample. The average recovery of the efavirenz in spiked samples was 69 ± 2% and the recovery of IS was 71 ± 2.3 % at the concentration used in the assay (30 ng/mL). Recoveries of the analyte and IS were good and consistent, precise and reproducible and agree with FDA guidelines.

3. A.5. Assay performance and validation

3. A.5.1. Selectivity and specificity

Representative chromatograms obtained from blank plasma, blank plasma spiked with IS, plasma spiked with lower limit of quantification with IS, and efavirenz spiked to plasma with higher concentration are shown in Figure 3A.2 (a- c). The mean % interference observed at the retention time of analytes or IS between 6 different lots of human plasma were found to be 0.00 %, which was within the acceptance criteria. Six replicates of extracted samples at the LLOQ level of EFV were prepared and analyzed. No interfering peaks were observed in the retention time of EFV. The % CV of the area ratios of these six replicates of samples were 0.2% for efavirenz confirming that interference does not affect the quantification at the LLOQ level. Utilization of selected product ions for each compound enhanced mass spectrometric selectivity. The product ions of m/z 316 to 244 were concluded to be specific for efavirenz.
Fig 3A.2 (a) Shows the typical chromatograms of a blank plasma sample (BL), IS spiked to blank sample (BL+IS),
Fig 3A. 2(b) Shows the representative ion chromatogram for (LLOQ 0.03 µg/mL)
Fig 3A.2 (c) Shows the Typical chromatogram shows the retention time of efavirenz and duloxetine
3. A.5.2. Linearity and LLOQ

The ratios of peak area of efavirenz to that of the internal standard duloxetine in the human plasma varied linearity over the concentration range of 0.03–4.57 µg/mL. Typical equations for the calibration curve was $Y=1.11x + -0.00385$ ($r = 0.9993$). In this equation, $Y$ is the mean peak area; $x$ is the concentration of EFV in µg/mL and ‘r’ is the correlation coefficient.

The LLOQ for efavirenz was 0.03 µg/mL. The intra-run precision and intra-run accuracy (% accuracy) of the LLOQ plasma samples containing efavirenz were 10.3% and 90.60%, respectively. Under the experimental conditions, the values obtained below 0.03 µg/mL for efavirenz were excluded from statistical analysis as they were below the LLOQ values.

3. A.5.3. Precision and accuracy

The inter-run precision and accuracy were determined by pooling all individual assay results of replicate (n=6) quality control over three separate batch runs analyzed on three different days. The inter-run precision (% CV) and inter-run accuracy as the % deviation between nominal and measured concentrations at LLOQ level were 10.30% and 97.00%, respectively for efavirenz.

The intra-run precision and accuracy were determined by pooling all individual assay results of replicate (n=6) quality control of two separate batch runs analyzed on the same day. The intra-run precision (% CV) and intra-run accuracy (the % deviation between nominal and measured concentrations) at LLOQ level were 12.12% and 103.12%, respectively. The intra-day and inter-day precision and accuracy for efavirenz are summarized in the Table 3A.2
Table 3A.2. Precision and accuracy for determination of efavirenz in human plasma

<table>
<thead>
<tr>
<th>Nominal concentration (µg/mL)</th>
<th>Within run (n=6)</th>
<th>% Recovery*</th>
<th>Between run (n=6)</th>
<th>% Recovery**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured concentration (µg/mL)</td>
<td>Mean ± SD (RSD)</td>
<td>Measured concentration (µg/mL)</td>
<td>Mean ± SD (RSD)</td>
</tr>
<tr>
<td>0.03</td>
<td>0.029 ± 0.003 (10.30 %)</td>
<td>97.00</td>
<td>0.033 ± 0.004 (12.12 %)</td>
<td>103.1</td>
</tr>
<tr>
<td>0.08</td>
<td>0.089 ± 0.005 (5.62 %)</td>
<td>107.20</td>
<td>0.086 ± 0.009 (10.46 %)</td>
<td>104.2</td>
</tr>
<tr>
<td>1.40</td>
<td>1.30 ± 0.048 (3.68 %)</td>
<td>94.98</td>
<td>1.36 ± 0.11 (8.07 %)</td>
<td>99.5</td>
</tr>
<tr>
<td>3.5</td>
<td>3.32 ± 0.05 (1.50 %)</td>
<td>96.77</td>
<td>3.21 ± 0.21 (6.52 %)</td>
<td>93.6</td>
</tr>
</tbody>
</table>

*Average of six determination.

**Average of six determinations.

3. A.5.4. Extraction recovery

The extraction recoveries of low, medium and high concentrations were 71.2 ± 1.8 %, 68.25 ± 2.1 % and 70.25 ± 3.4 %, respectively. The mean recovery for efavirenz was 69.9 % with a precision of 2.0% this indicates that the recovery for efavirenz as well, consistent and reproducible. Recovery of IS was 71 ± 2.3 % at the concentration used for the assay of (30 ng/mL). The recovery of the analyte from human plasma is in agreement with the FDA guidelines.

3. A.5.5. Stability studies

Bench-top, auto sampler stability for efavirenz was investigated at LQC and HQC levels. The results revealed that efavirenz was stable in plasma for at least 9 h at room temperature, and 24 h in an autosampler at 10 °C. It was confirmed that repeated freezing and thawing (four cycles) of plasma samples spiked with efavirenz at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that efavirenz was stable in matrix up to 53 days at a storage temperature of −50 °C. The results obtained from all these stability studies are tabulated in Table 3A.3.
The stock solution stability was also assessed at short stock solution stability (30 h), long term stock solution stability (20 days), and working solution stability (30 h). The results suggest that the analyte efavirenz was stable in the reconstituted solvent when extracts were stored at room temperature for at least 30 h.

Table 3A.3. Stability of efavirenz in human plasma samples (n=6)

<table>
<thead>
<tr>
<th>Stability parameter</th>
<th>Sample concentration (µg/mL)</th>
<th>Concentration found (µg/mL)</th>
<th>Mean ±SD</th>
<th>(%) CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bench top stability</td>
<td>0.08</td>
<td>0.07</td>
<td>0.003</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>3.43</td>
<td>3.32</td>
<td>0.165</td>
<td>4.96</td>
</tr>
<tr>
<td>Freeze thaw stability</td>
<td>0.08</td>
<td>0.07</td>
<td>0.002</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>3.43</td>
<td>3.32</td>
<td>0.20</td>
<td>6.07</td>
</tr>
<tr>
<td>In-Inj stability</td>
<td>0.08</td>
<td>0.08</td>
<td>0.00</td>
<td>10.59</td>
</tr>
<tr>
<td></td>
<td>3.43</td>
<td>3.44</td>
<td>0.35</td>
<td>10.17</td>
</tr>
<tr>
<td>Long term stability</td>
<td>0.08</td>
<td>0.07</td>
<td>0.01</td>
<td>13.20</td>
</tr>
<tr>
<td></td>
<td>3.43</td>
<td>3.28</td>
<td>0.45</td>
<td>13.75</td>
</tr>
</tbody>
</table>

3.A.6. Applications

The developed LC-MS/MS method was applied to the determination of efavirenz in human plasma. Each plasma sample was chromatographed within 3.5 min. This method is to be highly selective and suitable for bioavailability and bioequivalence studies of different formulations containing efavirenz formulations.
Section-3B

Bio analytical method development of an anti-HIV-1 drug efavirenz using high performance liquid chromatography

3B.1. Introduction

In the modern analytical laboratory, chromatographic techniques have been used extensively in the qualitative and quantitative analysis of bioactive compounds in pure and in pharmaceutical dosage forms. High performance liquid chromatography [HPLC] is inherently suitable for drug abuse analysis, because it easily and directly handles polar materials and readily quantitated by detector response. In addition, since compounds, which are ionic, non volatile or thermally labile, can be analyzed by HPLC, derivatization prior to chromatographic separation is not usually needed.

In the literature survey presented in section 3.2, several HPLC methods are presented for the estimation of EFV in tablets or in combination with other drugs or alone in biological fluids. In this section, the author has presented the development and validated stability indicating HPLC method for EFV in CPDA plasma. The method developed is simple, rapid, sensitive and cost effective method.

3B.2. Experimental

3B.2.1. Materials

Sodium dihydrogen ortho phosphate reagent grade obtained from Merck. Sodium hydroxide and sodium carbonate were purchased from Qualigens Fine Chemicals. Mumbai. Acetonitrile, methanol, ethyl acetate and n-hexane were purchased from Merck, Mumbai. Efavirenz drug substance was obtained from Wockhardt Research Center (Aurangabad, India) and Duloxetine (IS) was obtained from Cipla Ltd, Bangalore.
3B.2.2. Preparation of Reagents

50 mM sodium dihydrogen orthophosphate solution:
Dissolved 7.910 g of sodium dihydrogen orthophosphate in 1000 mL of milli-Q water and the pH was adjusted to 5.5 using sodium hydroxide solution.

Mobile phase [Acetonitrile and 50 mM sodium dihydrogen orthophosphate solution (pH-5.5) (48:52, v/v)]: It was prepared by mixing 520 mL of sodium dihydrogen orthophosphate buffer solution with 480 mL of acetonitrile, shaken well and filtered through a 0.2 μm nylon membrane filter. This solution was degassed in an ultrasonic bath for 3 min before its use.

Extraction solvent (ethyl acetate and n-hexane, 60:40, v/v): Prepared by mixing 300 mL of ethyl acetate and 200 mL of n-hexane in a reagent bottle and mixed well.

100 mM sodium carbonate: Accurately weighed 0.53 g of anhydrous sodium carbonate was dissolved in and diluted to mark in 50 mL standard flask with milli-Q water, mixed well and sonicated for 3 min.

Methanol: milli-Q water (1:1, v/v): Prepared by diluting 100 mL of methanol in 100 mL of milli-Q water. This solution was used as diluents in all subsequent preparation of the sample.

Standard solutions of efavirenz and duloxetine: Accurately weighed 10.367 mg of pure EFV was dissolved in and diluted to mark in a 10 mL standard flask with methanol. Dissolved 10.2 mg of IS in 10 mL standard flask with methanol. From the stock solution, working standards having concentration 1.54, 3.07, 10.97, 21.94, 54.85, 109.69, 182.82 and 228.53 μg/mL of efavirenz were prepared by suitable dilution with methanol:water (1:1 v/v). A volume of 15 μg/mL IS concentration was prepared by a diluent, methanol:water (1:1 v/v).

3B.2.3. Operating conditions and equipment

Column : A stainless steel .BDS Hypersil C18 (15cm.4.6mm, 5μm)
Mobile phase : Acetonitrile and 50 mM sodium dihydrogen orthophosphate solution (pH 5.5) (48:52v/v)
Solvent: HPLC grade acetonitrile

Column oven temperature: 30 °C

Flow rate: 0.8 mL/min

Detector wavelength: 247 nm

Injection volume: 15 µL

Total run time: 15 minutes.

Sample cooler temperature: 10 °C

Mode: Reverse phase

Shimadzu HPLC system model LC-2010 with Shimadzu tunable detector was used for chromatographic analysis. Nitrogen evaporator, pH meter, Vortex mixer, vibramax, refrigerated centrifuge and analytical balance were used.

3B.2.4. Construction of calibration curve

20 µL of each working standard solution was spiked into 980 µL of pooled plasma to give plasma concentration 0.03, 0.06, 0.22, 0.44, 1.1, 2.19, 3.66, 4.57 µg/mL. The extraction and reconstitution of efavirenz were done as per the method described in Section 3B.2.5. 15 µL of each of the above reconstituted samples (0.03 to 4.57 µg/mL in plasma) were injected and peak area was obtained. A calibration curve was constructed by plotting peak areas versus concentrations of efavirenz was drawn which serves as the calibration graph [Fig3B.1]. The quality control (QC) samples were separately prepared for assay in human plasma to get plasma spiked concentration of QC-Low=0.083, QC-Medium=1.37, QC-High=3.4 µg/mL, respectively. For blank keep separate pooled plasma without adding drug. The spiked plasma samples for calibrations, quality control samples and blank plasma were stored in freezer until analysis, and then used for on each analytical batch along with the unknown samples.
Fig. 3B.1 Calibration graph of EFV (µg/mL)
3B.2.5. Sample preparation

A 50 µL of IS and 250 µL of Na$_2$CO$_3$ (100 mM ) were added to 500 µL of plasma sample in 10 mL glass tubes and the mixture was vortexed for 10 min. The sample was extracted with 2.5 mL of ethyl acetate and n-hexane (60:40 v/v) by vortex mixing, and then it was centrifuged at 4500 rpm for 5 min. A volume of 2.0 mL of organic layer was then transferred into another glass tube and evaporated to dryness at 40 °C for 15 min. The residue was reconstituted with 500 µL of mobile phase, followed by vortexing and centrifuged for 10 min (4500 rpm). An aliquot of 15 µL was injected into the HPLC system for analysis.

3B.3. Bio-analytical method validation

The method was validated for accuracy, precision, sensitivity, recovery, linearity, and stability in accordance with FDA guidelines [36], using duloxetine as IS. Linearity, precision, and accuracy were tested by analysis of three batches of spiked plasma quality-control (QC) samples. Each batch of spiked plasma samples included one complete set of calibration standards (comprising eight different concentrations, blank, and blank with internal standard) and QC samples at low (0.083 µg/ml, LQC), middle (1.374 µg/mL, MQC), and high (3.436 µg/mL, HQC) concentrations.

Linearity was determined by regression analysis of the calibration data and determination of the coefficient of variation. Sensitivity was determined as the limits of detection (LOD) and quantification (LOQ). The accuracy of the method was evaluated as the EFV-to-IS peak-area ratio. Precision was measured as the coefficient of variation (%) for the low, middle, and high QC samples during validation. Recovery (%) was determined by measuring the EFV peak-area for the plasma QC samples (LQC, MQC, and HQC) with the EFV peak-area of freshly prepared unextracted aqueous standards containing the same concentrations of EFV. To determine the freeze–thaw stability of spiked plasma samples six replicates each of the low and high QC samples were analyzed after three freeze–thaw cycles. The freeze–thaw QC samples were quantified by comparison with a calibration plot prepared after chromatography of freshly spiked samples. For assessment of benchtop stability, the concentrations of low and high QC samples were determined after 8 h. The EFV-to-IS peak-area response at 8 h was compared with that after 0 h.
3B.4. Results and discussion

BDS hypersil C18 (15cm.4.6mm, 5μm), a reverse phase column is used for separation of a large number of organic compounds including some bioactive compounds. It was observed that this column gives satisfactory separation of efavirenz and duloxetine (IS) in the solvent system comprising of 50 mM sodium dihydrogen orthophosphate solution (pH 5.5) and acetonitrile.

3B.4.1. Mobile phase

The various compositions of the mobile phases consisting of phosphate buffer-acetonitrile were investigated; a mobile phase consisting of phosphate buffer-acetonitrile (60+40) at pH 4.5 did not give the sensitive results. The use of mobile phase consisting of phosphate buffer and acetonitrile (55+45) adjusted to pH 6.5 did not yield good separation within a short time. A good separation was obtained by the mobile phase consisting of acetonitrile and phosphate buffer.

Finally, a mobile phase of acetonitrile and 50 mM sodium di-hydrogen orthophosphate solution (pH 5.5) (48:52, v/v) gave the optimum resolution of efavirenz and duloxetine. No interference in plasma samples in analyte and IS, retention time was encountered using this mobile phase. The pH of mobile phase was a critical factor in obtaining good resolution and sharp peaks. It also influenced the retention times of the two components. The concentration of the di-hydrogen orthophosphate buffer only influenced the retention times of the efavirenz and duloxetine.

3B.4.2. Flow rate

The flow rate of mobile phase also checked with 0.5, 0.7, 0.8, 1.2, and 1.0 mL/min. In 0.8 mL/min flow to get sharp and symmetrical peaks with good resolution.

3B.4.3. Retention times

The reproducibility of the retention times of efavirenz and duloxetine were assessed by injecting six aqueous standards by preparing the mixture of 40 μL of
analyte and 200 µL of IS dilute to 2.0 mL with mobile phase. The mean retention times of six injections of both analyte and IS %CV should be within 0.5%.

3B.4.4. Linearity of the detector response

Various aliquots of standard solution were mixed with fixed amount of an internal standard and diluted to 2.0 mL. Then, 15 µL of each solution was injected on to the column. The concentration of efavirenz was plotted against the ratio of its peak area to that of IS (Fig. 3B.1). Linearity between detection response and concentration was observed in the range of 0.03 to 4.57 µg/mL.

3. B.5. Assay performance and validation

3. B.5.1. Selectivity and specificity

Representative chromatograms of blank and blank plasma spiked to IS are illustrated in chromatogram. Blank plasma showed very less interference at the retention time of analyte and internal standard. Fig. 3B.2 (a-d) shows the typical chromatograms of a blank plasma sample (BL), IS spiked to blank sample (BL+IS), blank plasma sample spiked with efavirenz at the LLOQ and IS. And typical chromatogram shows the retention time of efavirenz and duloxetine was 10.5 and 4.15 min, respectively. No significant interference from endogenous substances with analyte or IS was detected.
Fig. 3B.2 (a) typical chromatograms of a blank plasma sample (BL),
Fig. 3B.2 (b) blank sample spiked to IS (BL+IS),
Fig. 3B.2(c) blank plasma sample spiked with efavirenz at the LLOQ and IS.
Fig. 3B.2(d) Typical chromatogram shows the retention time of efavirenz and duloxetine
3. B.5.2. Linearity and LLOQ

The calibration curve was obtained by plotting the peak area ratio of EFV to internal standard versus nominal concentration of EFV. For linearity study eight different concentrations of EFV were analyzed (0.03, 0.06, 0.21, 0.43, 1.1, 2.19, 3.65 and 4.57 µg/mL). The peak area response was linear over the concentration range studied. At each concentration, the experiment was repeated three times on three separate days to obtain the calibration data. The coefficient of correlation r was found to be 0.999. The limit of quantification was 0.03 µg/mL.

For efavirenz, a concentration of 0.03 µg/mL was defined as the limit of quantification. A representative chromatogram of spiked plasma at the limit of quantification is shown in the Fig. 3B.2c

3. B.5.3. Precision and accuracy

The results from the validation of the method in human CPDA plasma are listed in Table. 3B.2. These results indicated that the method is accurate, precise, within day precision ranged from 0.07-2.47%. Between run precision ranged from 5.96 - 12.2%, and accuracy ranged from 96.43 to 112.71%.

<table>
<thead>
<tr>
<th>Nominal concentration (µg/mL)</th>
<th>Measured concentration (µg/mL)</th>
<th>% Recovery*</th>
<th>Between run (n=3)</th>
<th>% Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (RSD)</td>
<td></td>
<td>Measured concentration (µg/mL)</td>
<td>Mean± SD (RSD)</td>
</tr>
<tr>
<td>0.03</td>
<td>0.029 ± 0.0007 (2.47 %)</td>
<td>96.66</td>
<td>0.03 ± 0.004 (12.2 %)</td>
<td>110.01</td>
</tr>
<tr>
<td>0.08</td>
<td>0.085 ± 0.0016 (1.91 %)</td>
<td>101.20</td>
<td>0.08 ± 0.009 (11.1 %)</td>
<td>96.43</td>
</tr>
<tr>
<td>1.37</td>
<td>1.46 ± 0.0019 (0.13 %)</td>
<td>106.02</td>
<td>1.35 ± 0.11 (8.13 %)</td>
<td>98.65</td>
</tr>
<tr>
<td>3.44</td>
<td>3.87 ± 0.003 (0.07 %)</td>
<td>112.71</td>
<td>3.52 ± 0.21 (5.96 %)</td>
<td>102.47</td>
</tr>
</tbody>
</table>

*Average of six determinations
3. B.5.4. Extraction recovery

The extraction recoveries of low, medium and high concentrations were 65.23 ± 2.3 %, 68.76 ± 4.1 % and 67.11 ± 4.8 %, respectively. Overall recovery of three level was 69.9 ± 2 % and the recovery of IS was 71 ± 2.3 % at the concentration used in the assay (15 μg/mL).

3. B.5.5. Stability studies

The stability of EFV in plasma was determined by measuring concentration change in quality control samples over time. Stability was tested by subjecting the quality controls to three freeze-thaw cycles and compared with freshly prepared quality control samples, the mean concentration of EFV in quality control samples did not change significantly within the time period under the indicated storage conditions % CV < 6 in LQC and 4.2 in HQC levels. Bench top stability studies results conclude that EFV is stable in plasma matrix at least 10 h when opened in room temperature, there was no appreciable change, %CV is <5.7 and 6.2 for LQC and HQC levels, respectively. Auto sampler stability of plasma samples was examined by supplementing blank plasma with appropriate amounts of working solutions of EFV to yield quality-control (QC) samples containing 0.08, 1.37, and 3.43 μg/mL. Each sample was stored at 10°C for 48 h in auto sampler, % CV values were: 1.84, 1.50 and 1.67% for EFV concentrations, respectively.

3B.6. Applications

The developed HPLC method was applied to the determination of efavirenz in human plasma. The experimental results of the assay of efavirenz is reliable, the retention time of efavirenz was 10.44 min and that of IS was 4.15 min. Response was linear over the concentration range of 0.03-4.57 μg/mL in human CPDA plasma. The method was simple, precise, accurate and reproducible.

Conclusion

Although several methods are available in the literature for the determination of an anti-HIV drug efavirenz in its commercial as well as in real samples, the developed methods are the first assay for the measurement of efavirenz in human
CPDA plasma, which is cheaper than K$_2$EDTA and sodium fluoride potassium oxalate anticoagulant that integrates both the high sensitivity and simplicity. The newly developed assay was successfully applied to the human pharmacokinetic study of efavirenz, and has the potential to accurately estimate the pharmacokinetic parameters of efavirenz with more confidence.

Liquid-liquid extraction technique used for sample preparation, run time also less than 3.5 minute per sample. This makes it good procedure for high through output bio analysis of efavirenz in plasma samples. Current method has shown acceptable precision and accuracy for quantification of efavirenz in plasma samples for pharmacokinetic and bioequivalence studies.
References


