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Research Article

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A New Method for Determination of Efavirenz and pK_a by Using LC-UV

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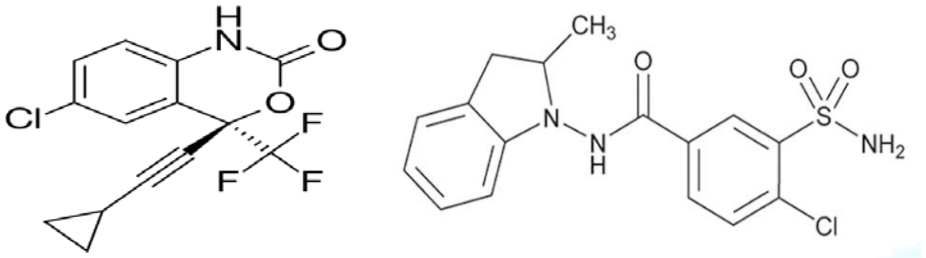
ABSTRACT

Efavirenz (EFA) is a non-nucleoside reverse transcript-inhibitor (NNRTI) and clinically, it is used as a part of a highly active antiretroviral therapy for the treatment of a human immunodeficiency virus. This research has been concerned with the determination of pKa values and the development of simple, specific, accurate and reproducible isocratic reversed phase high performance liquid chromatographic (RP-HPLC) method which has been subsequently validated using ICH recommendations for the determination of Efavirenz (EFA) in tablet dosage forms. Indapamide (IDP) was used as an internal standard and it was the first of the new class of antihypertensive diuretic. The determination was carried out for a runtime of 8 min at 30˚C on a X-terra RP-18 column having 250mm x 4.6mm i.d. with 5µm particle size and 20 mM sodium dihydrogen phosphate buffer adjusted to pH 5.0, and acetonitrile (70:30 v/v) was used as a mobile phase at a constant flow rate of 1.0 ml/min with UV detection wavelength of 247 nm for EFA and 240 nm for IDP. The retention time of IDP and EFA was about 3.6 and 6.9 min with the correlation coefficient of 0.999 and 0.999; respectively. The linearity was established at 1-12 µg/ml for EFA, and the LOD and LOQ values were found to be 0.083 and 0.261 with the recovery of 100.06%. The pK_a values of EFA have been determined precisely in different acetonitrile–water binary mixtures (60-65 % v/v) using retention factors. The method developed in this study can be used in routine analysis of EFA in pharmaceutical quality control laboratories and pharmacokinetic studies.

Key words: *Efavirenz, pK_a Determination, Validation, Dosage Form, LC-UV.*

INTRODUCTION

Efavirenz (EFA) is a non-nucleoside reverse transcript-inhibitor (NNRTI), and clinically it has been used as part of a highly active antiretroviral therapy for the treatment of the human immunodeficiency virus (HIV) [1]. The chemical structure of EFA is (S)-6-chloro- (cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-,1-benzoxazin-2-one (Figure 1 a). EFA is currently one of the most widely used non-nucleoside reverse transcriptase inhibitors by both adults and children [2]. Indapamide (IDP), 4-chloro-N (2 methyl-2,3-dihydroindol-1-yl)- 3-sulfamoyl benzamide has been as an internal standard, and it has been the first of the new class of antihypertensive diuretics which has been prescribed to treat the salt and fluid retention associated with the congestive heart failure [3] (Figure 1 b).



(a) (b)

Figure 1. Chemical structure of EFA (a) and IDP (b)

Several chromatographic techniques such as HPLC [4-10], LC/MS detection [9-13] or UPLC/MS-MS [14] and capillary electrophoretic methods [15] have been used for the analysis of EFA for different samples. Among the chromatographic method, LC-MS/MS method has been used commonly because of very low detection limits, but this system may not be available because of the price. Most of the methods in the literature have used expensive equipment, such as solid-phase extraction, which is not suitable for the routine pharmaceutical and pharmacokinetics analysis. However, these methods require complex instrumentation and radioactive chemicals, which can lead to the time loss. HPLC has been widely used for the regular analysis of EFA, so it has been necessary to develop a simple, novel, validated and rapid HPLC method for it.

The absorption, metabolism, distribution, toxicity and excretion are the important parameters of a drug molecule, and the dissociation constant (pK_a) manages the distribution, elimination of substances, solubility and absorption [16]. In addition, pK_a values are important data to fully understand some chemical phenomena such as biological uptake and binding of these molecules to environmental matrices, and the formation of chelates with metallic cations [17]. Sufficient information on the acid-based behavior of the substances in hydro-organic media such as acetonitrile (MeCN) - water has been necessary to estimate the effect of pH on the selectivity and retention during HPLC [18, 19]. Until this time, no detailed report on the determination of pK_a values of EFA by LC has been provided.

In this study, pK_a values of EFA were determined in two different MeCN-water binary mixtures. The experimental region was selected as 60-65 % (v/v) MeCN, because under these percentages, EFA could not be eluted from the LC column. On the other hand, new, simple and fast LC method for the determination of EFA was developed and fully validated using ICH recommendations. This method was used for the determination of EFA in its pharmaceutical dosage forms. IDP was chosen for the internal standard because of the suitable retention time for the analysis.

MATERIALS AND METHODS

Chemical and Reagents:

All chemicals and solvents were of analytical reagent grade, and used without further purification. EFA and IDP were purchased from Sigma. HPLC grade acetonitrile, (MeCN) was purchased from Merck. Sodium dihydrogen phosphate (NaH_2PO_4), ortho-phosphoric acid (H_3PO_4), sodium hydroxide (NaOH), hydrochloric acid (HCl), uracil and methanol (MeOH) were obtained from Sigma-Aldrich (St. Louis MO, USA). Hydrochloric acid (Titrisol) and potassium hydrogen phthalate (dried at 110°C before use, Sigma-Aldrich) were used. Ultrapure water, with conductivity lower than 0.05 µS/cm was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

MeOH was used for the stock standard solutions of EFA and IDP (100 µg/mL). They were diluted with the corresponding mobile phase to 10 µg/mL. All of the solutions were protected from light, and stored in fridge at about 4°C. For dead time, uracil solution was used. It was solved (0.01% (v/w)) in water, and injected for each studied mobile phase and pH.

Apparatus:

The LC analysis was carried out on an Agilent 1260 series. HPLC system with ternary solvent pump, online degasser, automatic injection system, column heater and multi wavelength detector was used. UV detection was performed at 247 nm and 240 nm for EFA and IDP; respectively. The flow rate was 1 $mL.min^{-1}$ for all the

analyses. A X-terra RP-18 (250 x 4.60 mm i.d. x 5 µm) column was used as a stationary phase at 30°C. Mettler Toledo MA 235 pH/ion analyzer with Hanna HI 1332 Ag/AgCl combined glass electrode was used for pH measurements. The internal standard (IDP) was used for EFA analysis.

Chromatographic Procedure:

In this study for pK_a determination of EFA, two different MeCN-water binary mixture (60% and 65% v/v) containing 20 mM NaH_2PO_4 were used. The pH of the mobile phase was adjusted between 2 and 11.5 by the addition of appropriate amount of sodium hydroxide or hydrochloric acid. The flow rate during the analysis was kept constant at 1.0 mL/min. The injection volume was 10 µL. For pK_a calculations, three injections of EFA were performed. Then, the retention time values were obtained for every pH values. After having retention time values, the capacity factors were calculated using the expression of $k = (t_R - t_0)/t_0$. For the determination of dead time (t_0), the uracil solution was injected for each studied mobile phase composition and pH. The pK_a values of EFA were calculated by using non linear regression program NLREG [20]. This was a general purpose program, where the function to be minimized and the parameters to be estimated can be defined by means of the built in program editor. NLREG refined these parameters according to equation (1) to give a minimum in the sum of square residuals (U_m) in order to obtain the dissociation constants of the compounds studied.

$$U_m = \sum_{j=1}^{n_s} (k_{i,\exp} - k_{i,calc})^2 \quad (1)$$

where n_s indicates the number of solutions, $k_{i,\exp}$ is the experimental value of the retention factor for solution, i, and $k_{i,calc}$ are the calculated value.

Analysis of Pharmaceutical Preparations:

Ten tablets of Stocrin® (containing 600 mg EFA, Patheon Int. Canada), were weighted and powdered. The required amount of this powder, equivalent to a stock solution of 10 mg/L EFA was weighted and transferred into a 100 mL volumetric flask, and diluted with MeOH. This solution of EFA was sonicated for 15 min to complete its dissolution. After the filtration of the solution, the internal standard was added at the desired concentration, and the final solution concentrations were reached by diluting this solution with the mobile phase. The amount of active drug in the tablet was calculated from the calibration graph plotted using pure drug actives. The recovery values were then examined to check the accuracy of the improved method, and investigate the interaction of the additives in the drug. For this purpose, the known amount of the EFA pure standard was added to the tablet formulation. This analysis was repeated five times, and the recovery value was calculated for EFA by using calibration graph.

RESULTS AND DISCUSSION

Determination of dissociation constants (pK_a):

Most of the antiviral drugs contain both proton acceptor and proton donor groups, which may be ionized and/or protonated. The percentage of the ionized and non-ionized species of each compound has been the most important factor determining their retention on the column. The biggest change in the retention was realized at pH values within pK_a±1.5. The ionization values helped to determine the pH of the buffer to be used in the mobile phase. So that the chromatographic behavior of the studied compounds can be understood clearly. MeCN-water mixtures have been usually used as a mobile phase, so knowledge on the acid-base dissociation constants of the studied compounds can help to improve the separation conditions.

In the literature, there has been one data for pK_a of EFA which was determined previously by Rabel et al. using spectrophotometric and pH-solubility examinations, and it was found to be 10.1 ± 0.1, which was anomalous with respect to the typical ionization behavior of the cyclic carbamates [21].

Table 1 shows the dissociation constant of EFA obtained by means of LC using the non-linear regression program of NLREG in several MeCN–water mixtures (60 and 65 % (v/v)) and the literature values [18-20]. In Figure 2, the data pairs of k/pH for EFA in 60% (v/v) MeCN have been shown, and the correlation between the experimental capacity factors of the EFA studied over the whole experimental pH range was good.

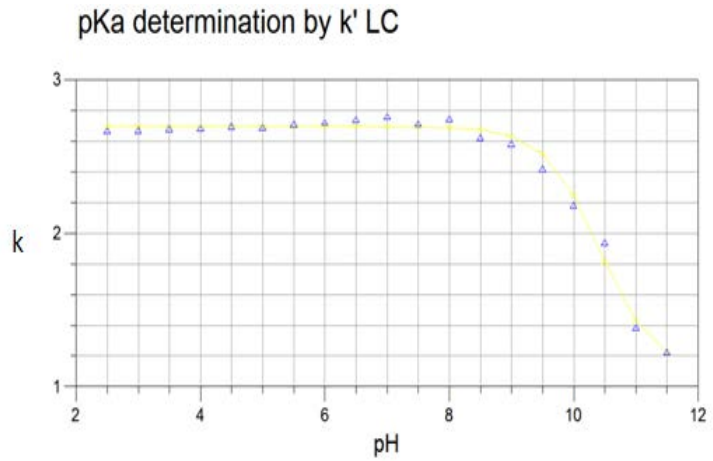


Figure 2. NLREG graphic of EFA in 60% (v/v) MeCN-water binary mixture

Table 1. The pK_a values of EFA, obtained by LC method in MeCN–water media at 30 ºC.

Compound	Literature value	ACD Lab	SPARC	60% (v/v) MeCN	65% (v/v) MeCN
EFA	10.1±0.1 [21]	7.92±0.40 [22]	6.74 [23]	10.40 ± 0.05	10.38 ± 0.08

The most important factor determining the dissociation constants has been the reaction medium. The pK_a values of EFA decreased with the increasing percentage of MeCN.

Analysis of commercial samples:

Three mobile phase systems were used to provide an appropriate LC separation (MeCN-water mixtures at 60, 65 and 70 % v/v). Four different types of columns such as Ascentis RP Amide (3.5 µm, 150 mm x 4.6 mm ID), Gemini C18 110 Aº (250mm x 4.6mm x 3µm), YMC Pack ODS-AM (5 µm, 250 mm x 4.6 mm ID) and X-Terra C-18 (5 µm, 250 mm x 4.6 mm ID) were tested in order to find the best resolution and peak shape of the EFA and IDP.

EFA could not be eluted from the column by using YMC Pack ODS-AM (5 µm, 250 mm x 4.6 mm ID) and Gemini C-18 (3 µm, 250 mm x 4.6 mm ID). Optimum separation conditions (peak shapes, retention times etc.) were obtained by using X Terra C-18 column as a stationary phase. This column had a thermally more stable, and expanded pH value as compared to the normal silica-based columns.

For the optimum chromatographic conditions, the influence of pH on the mobile phase and the column temperature were examined on the separation of EFA and IDP. pH 5.0 and 30°C were selected as the optimum values which were optimum pH with the best peak asymmetry and retention values with the aid of the determined pK_a values. As a result, the mobile phase MeCN: water 70: 30 (v/v) with 20 mM NaH_2PO_4 (pH 5) at a flow rate of 1.0 mL/min was chosen as the most suitable for EFA and IDP.

The proposed RP-LC method provided a simple procedure for these compounds in drug formulations at 247 nm for EFA and 240 nm for IDP. In these conditions, the separation of these two compounds was performed in a short analysis time (< 8 min.).

System suitability tests were used to verify the reproducibility of the developed method. Some of these tests were performed using freshly prepared solutions. Tailing factors of 0.85 and 0.87 were obtained for EFA and IDP; respectively. The theoretical plate numbers (N) were 12320 for EFA and 7358 for IDP. The selectivity factor was 3.089. The retention times of IDP and EFA were 3.683 and 6.985 min; respectively.

The calibration curve was obtained by linear least squares regression. The concentration of IS was maintained at a constant level of 4 mg/L. The linearity was calculated by plotting the peak area ratio of drug to IS vs. the concentration of the compound. The developed RP-LC method was also validated according to the standard procedures, and the results were reported in Table 2. The method exhibited good linearity based on a correlation coefficient > 0.999. The LOD and LOQ were calculated as LOD = 3.3 s m^{-1} and LOQ = 10 s m^{-1}, where s is the standard deviation of the response and m is the slope of the corresponding calibration curve.

Table 2. Statistical evaluation of the calibration data of EFA by RP–HPLC

	EFA
Linearity range (µg/ml)	1-12
Slope	0.262
Intercept	-0.1122
Correlation coefficient (*r*)	0.999
Detection limit (LOD) (µg/ml)	0.083
Quantitation limit (LOQ) (µg/ml)	0.261
Number of points	5

The repetitive analyses of the EFA were performed to calculate the accuracy and precision of the method. In the characterization of the reproducibility and repeatability values, the mean recovery and RSD values were used, and the results have been summarized in Table 3. As seen from Table 3, the results supported good precision of the method.

Table 3. EFA assay results and the recovery analysis in pharmaceutical dosage forms

	EFA
Labeled claim (mg)	600.00
Amount found (mg)[a]	600.085
RSD (%)	0.556
Bias (%)	-0.085
Added (mg)	300.000
Found (mg)[a]	300.078
Recovery (%)	100.055
RSD (%)	0.498
Bias (%)	-0.078

[a] Each value of the mean five experiments.

The adequacy of the developed method was evaluated as a result of the analysis of EFA from the tablet forms (labeled values 600 mg). This developed method can be used for the assay of EFA in several samples. The concentration of the drug determined in tablets has been reported in Table 3. The data obtained at the end of the study showed that the method can be applied successfully in the determination of EFA in tablet without any interference (Table 3). The chromatogram of tablet samples has been given in Figure 3.

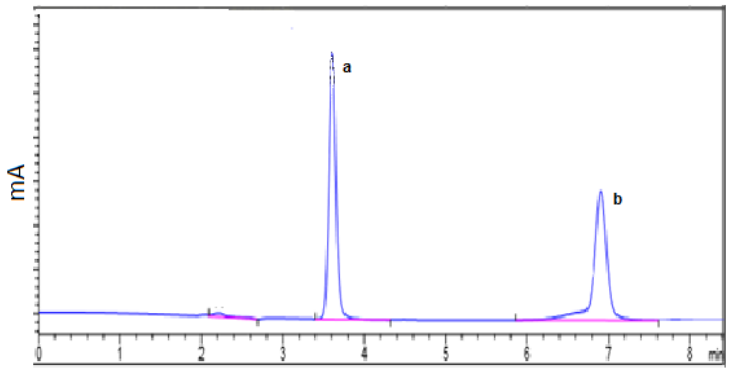


Figure 3. Representative chromatogram obtained from tablet dosage forms under optimum conditions (a) Indapimide (b) Efavirenz

The recovery studies have been performed to determine the accuracy and precision of this method. In the recovery studies, the known concentration from the EFA standard was spiked to analyze the tablet samples. The recovery analysis result has been given in Table 3. As a result, the sufficient precision and accuracy could be achieved to by this developed method for the analysis of EFA in pharmaceutical dosage forms.

The determination of pK_a value of EFA in MeCN-water binary mixture was done for the first time by the liquid chromatography in this study. Many properties of the compounds such as solubility, permeability, lipophilicity were dependent on pK_a values. The pK_a value can also affect the drug-receptor binding. Therefore, pK_a value of EFA was determined and reported. Also, the developed method for the determination of EFA in tablets, provided a sensitive, simple, rapid, experimentally convenient, cost-effective and high-throughput approach for

the determination of pK_a value of EFA, without the necessity of the sample pretreatment, or any time-consuming evaporation or extraction steps prior to the analysis. By this method, EFA analysis could be completed about 8 minutes setting to the instrument parameters by RP-LC method.

CONCLUSION

This study has been the first study dealing with the determination of pK_a values of EFA by RP-LC method in MeCN–water binary mixtures. Determining the pK_a value of a compound provided a means of calculating the solution ionization state, and also provided information for the pharmacokinetics and pharmacology studies such as absorption, distribution, metabolism, and excretion. Additionally, a new, reversed-phase HPLC method has been developed for the analysis of EFA in a tablet formulation. It was shown that, the method was linear, accurate, reproducible, repeatable, precise, selective and specific proving the reliability of the method. The run time was relatively short, i.e. 8 min, which enabled the rapid determination of any samples in routine and quality control analysis of the tablet formulations. No interference from any excipient was observed. Hence, the method might be employed for the quality control analysis as well as in further studies for other matrices, such as plasma. Efavirenz tablets analyzed by the validated method showed the adequate quality and drug contents in concordance with the labeled amount.

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Conflict of Interest

There was no conflict of interest between authors.

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