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VALIDATED STABILITY INDICATING LC-PDA-MS METHOD TO INVESTIGATE PH RATE PROFILE AND DEGRADATION KINETICS OF EFAVIRENZ AND IDENTIFICATION OF HYDROLYSIS PRODUCT BY LCMS

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Abstract

A rapid, selective and sensitive stability indicating liquid chromatography-photo diode array-mass spectrometry (LC-PDA-MS) assay was developed and validated for the quantitative analysis of efavirenz in presence of its degradation products. A 150 mm ODS column was used with mobile phase consisting of acetonitrile-ammonium acetate (100 mM) (60:40, v/v) as mobile phase. Quantification was achieved by UV detection at 246 nm, on the basis of peak area. Forced degradation studies were performed on a bulk sample of efavirenz using 0.1 M hydrochloric acid, 0.1M sodium hydroxide, 0.33% hydrogen peroxide, heat (70 °C), and photolytic degradation. The method was linear in the range of 0.5 – 60 µg mL^{-1} efavirenz concentration. Excellent recoveries (99.2 – 101.3 %) proved that the method was sufficiently accurate. The LOD and LOQ were found to be 50.0 and 160 ng mL^{-1}, respectively. Efavirenz found unstable in alkaline condition. The alkaline hydrolysis product of efavirenz was identified on single quadrupole mass spectrometer. The hydrolysis product was proposed from mass spectral data to be amino alcohol formed by hydrolysis of cyclic carbamate. The alkaline hydrolysis of drug followed apparent first order degradation kinetics. The half-life of drug in alkaline solution was found to be 69 h.

Keywords: Efavirenz, Stability indicating assay, Forced degradation, pH-Rate profile, Degradation kinetics, LC-PDA-MS

INTRODUCTION

Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as a part of highly active anti retroviral therapy (HAART) for the treatment of human immunodeficiency virus (HIV-1)[1]. Both nucleoside and non-nucleoside RTIs inhibit the same target. The reverse transcriptase enzyme transcribes viral RNA into DNA. Unlike nucleoside RTIs, which bind at the enzyme's active site, NNRTIs bind within a pocket, termed the NNRTI pocket. Efavirenz is not effective against HIV-2, as the pocket of the HIV-2 reverse transcriptase has a different structure, which confers intrinsic resistance to the NNRTI class[2-5].

Several analytical methods for efavirenz were reported including LCMS[6-7], HPLC [8]. A study of hydrolysis of efavirenz was also reported in literature; the hydrolysis product was confirmed with help of reference standards[9].

The International Conference on Harmonization (ICH) guideline entitled 'stability testing of new drug substances and products requires stress testing to be performed to elucidate the inherent stability characteristics of the active substance [10-11]. Hydrolytic, oxidative, thermal and photolytic stability should be determined. Because no method has been reported in the literature for simultaneous study of stability, kinetics and identification of hydrolysis product of Efavirenz, it was thought necessary to develop a versatile method for stability studies, identification of hydrolysis product by LCMS and to study degradation kinetics.

This paper describes selective and rapid analytical method, which enables simultaneous study of stability, degradation kinetics and identification of degradation products. The sample preparation and mobile phase preparation steps were made simpler which do not required any pH

adjustment. The total run time of method per sample was 6.0 min.

MATERIALS AND METHODS

Efavirenz was extracted from tablets. Purified water was obtained from Milli-Q Water purification system. Methanol (HPLC grade), Acetonitrile (HPLC grade) were purchased from Qualigens (India). Hydrochloric acid and sodium hydroxide both certified grades were purchased from spectrochem (India). Orthophosphoric acid, potassium chloride, potassium phosphate (mono basic), potassium phosphate (di basic), ammonium acetate (AR grade) were purchased from Qualigens (India). All reagents were used as received without further purification.

Extraction of efavirenz from tablets

Efavirenz tablet (500 mg) was crushed to powder and sonicated with 100 mL methanol for 5 min. The solution was filtered through 0.45 µm membrane filter then lyophilized for 12 h. The solid efavirenz obtained was approximately 99.9 % pure.

Chromatographic and mass spectrometric system

The HPLC system (LC2010, Shimadzu Corporation, Kyoto, Japan) consisted of low-pressure gradient quaternary pump, Auto sampler, column-oven and photo diode array detector (SPD M20A). LCSolution workstation was used for data acquisition. The analytical column used for the study was C18, 150 mm length, 4.6 mm ID and 5 µm particle size (YMC ODS A, Japan). LCMS analysis was performed using LCMS-2010 single quadrupole equipped with electrospray ionization interface (Shimadzu Corporation, Kyoto, Japan). The data were collected and processed using LCMS solution software.

An LCMS-2010 single quadrupole mass spectrometer was interfaced with electrospray ionization (ESI) probe. The temperatures were maintained at 250, 250 and 200 °C for the probe, CDL and block respectively. The voltages were set at 4.5 kV, -30 V, 25 V, 150 V, and 1.6 kV for the probe, CDL, Q-array 1, 2, 3 bias, Q-array radio frequency (RF) and detector, respectively. The flow rate of nebulizer gas and dried gas were set at 1.5 L min^{-1}.

The mobile phase consisted of 100 mM ammonium acetate and acetonitrile (40:60) (*v/v*). Prior to analysis the mobile phase was degassed using a Millipore vacuum pump. The detector was set at 246 nm. The runtime was set for 6 min. The flow rate was maintained to 1.2 mL min^{-1}. The column oven temperature was maintained to 30 °C. The injection volume was 10 µL.

Forced degradation study

Acid induced hydrolysis

The reaction was initiated by adding 5.0 mL of efavirenz (1.0 mg mL^{-1}) to 100 mL volumetric flask containing 0.1 M hydrochloric acid. The volume made to 100 mL with 0.1 M hydrochloric acid. The degradation was carried out in thermostatically controlled water bath, protected from light.

Alkali induced hydrolysis

The reaction was initiated by adding 5.0 mL of efavirenz (1.0 mg mL^{-1}) to 100 mL volumetric flask containing 0.1 M sodium hydroxide. The volume made to 100 mL with 0.1 M sodium hydroxide. The degradation was carried out in thermostatically controlled water bath, protected from light.

Effect of hydrogen peroxide

The reaction was initiated by adding 5.0 mL of efavirenz (1.0 mg mL^{-1}) to 100 mL volumetric flask containing 0.33 % hydrogen peroxide. The volume made to 100 mL with 0.33 % hydrogen peroxide.

The degradation was carried out in thermostatically controlled water bath, protected from light.

Effect of light

Efavirenz (50 mg, accurately weighed) was dissolved in 100 mL methanol. The solution was exposed to light from UV lamp for 24 hrs.

Effect of heat

The thermal stability of efavirenz was studied by heating it, both as powder and in methanolic solution (without pH adjustment) to 70 °C for 7 days.

Standard solution and calibration

Stock solution was prepared by dissolving 25 mg efavirenz in 25 mL methanol. Standard solutions were prepared by dilution of stock solution with mobile phase to furnish concentrations in the range 0.50 - 60 μg mL^{-1}. Triplicate 10 μL injections of each solution were chromatographed under the conditions described above. Peak areas were plotted against the corresponding concentrations to obtain calibration plot.

Kinetic investigation of alkaline hydrolysis

Efavirenz (25 mg accurately weighed) was dissolved in minimum amount of methanol and diluted to 250 mL with water. Separate 2 mL volumes of this solution were transferred to separate stoppered conical flasks and mixed with 2 mL 0.1 M sodium hydroxide. The flasks were placed in a thermostatic oven at different temperatures (30, 40, 50, 60, 70 °C) for 48 hours. The contents of the flasks were then neutralized to pH 7.0 with 0.1 M hydrochloric acid, transferred to 10-mL volumetric flasks, and diluted to volume with mobile phase. Each solution (10 μL) was chromatographed under the conditions described above and concentration of efavirenz remaining was then calculated for each temperature.

pH rate profile

For pH rate profile, phospate buffer solutions ranging in pH from 2 to 13 were used. The buffer concentration was 0.1 M. A constant ionic strength of 0.1 was maintained by adding appropriate amount of potassium chloride. All studies were conducted at 30 °C.

Identification of hydrolysis product

The HPLC method described above was used for separation of impurity on LCMS. The ionization done in positive mode with detector voltage 1.5 kV, nebulizer gas at 1.5 L/min, CDL temperature to 250 °C, heater block at 200 °C.

Results and Discussion

Chromatographic Conditions

The choice of chromatographic conditions selected was based on symmetry of peak shape and reduction of chromatographic analysis time. The chromatographic separation was achieved using a mobile phase containing a mixture of aqueous 0.1 M ammonium acetate and acetonitrile in the ratio (40:60, *v/v*). The column temperature (30 °C) has improved the peak shape of efavirenz. In optimized conditions, efavirenz and hydrolysis product were separated with a resolution greater than 2 and typical retention times of hydrolysis product and efavirenz were about 2.6 and 3.0 min (Fig 1), respectively. The system suitability results were given in Table 1 and the developed LC method was found to be specific for efavirenz and its hydrolysis products.

Table 1: System suitability report

Compound (*n*=3*).*	t_R	R_S	N	T
Efavirenz	3.0	2.07	4282	1.46

n =3 determinations, t_R = Retention time in minutes, Rs = USP Resolution, T = USP tailing factor. N = No. of theoretical plates

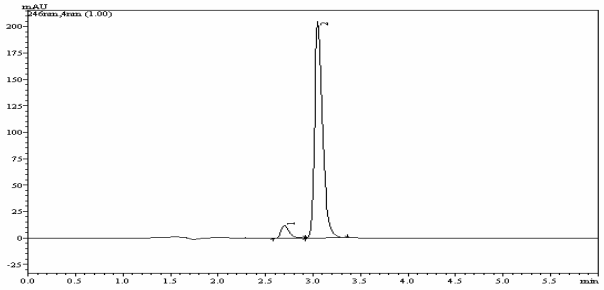

Fig. 1: Representative chromatogram obtained from alkaline hydrolysis solution of Efavirenz. Peaks: 1 = hydrolysis product, 2= efavirenz

Optimization of LC-MS Condition

The choice of ionization mode was guided by base peak with higher intensity in LC-MS analysis. The mass spectra of efavirenz and its hydrolysis product obtained from scan mode were characterized by a protonated molecular ion $[M+H]^+$ as base peak. To confirm ionization mode mass spectra were measured in ESI and APCI positive and negative mode. In both ionization modes the base peak intensity of positive ion was higher than those of negative ion, and the efficiency of ionization in ESI was higher than APCI. There fore ESI positive ionization mode selected for LCMS analysis and hydrolysis product identification.

Identification of Hydrolysis Product

The efavirenz structure contains cyclic carbamate group, this group was suspected to undergo alkaline hydrolysis. Efavirenz peak shows mass 315(M+1) and the hydrolysis product peak shows mass 290(M+1) in positive ionization mode (Fig 2), which confirms the hydrolysis of cyclic carbamate to the corresponding amino alcohol (Fig 3).

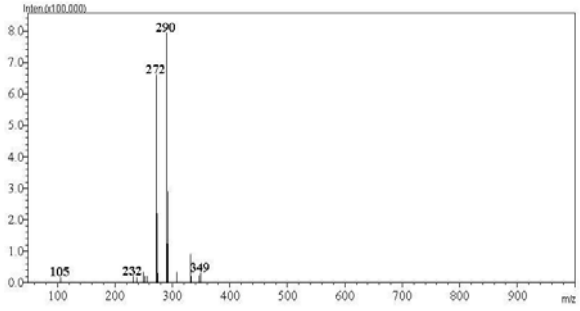

Fig. 2: Representative mass spectrum of Efavirenz hydrolysis product

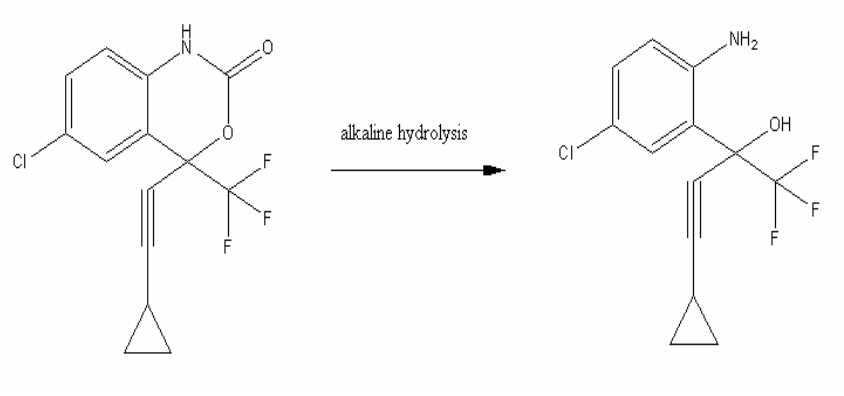

Fig. 3: Proposed pathway of hydrolysis of efavirenz in alkaline solution

Stability Indicating Assay

Degradation was not observed for efavirenz sample during stress conditions like heat, acid and oxidation except in base hydrolysis and UV light. Efavirenz was degraded during base hydrolysis and the structure of hydrolysis product was confirmed by LCMS. Peak purity test results confirmed efavirenz peak is homogeneous in all the stress conditions tested. The mass balance of efavirenz in test samples was close to 100% and moreover, the unaffected assay of efavirenz in presence of hydrolysis product confirms the stability indicating power of the method. The non-interference of excipient peaks with efavirenz confirms the specificity of the developed method in formulation samples. The summary of forced degradation studies is given in Table 2.

Kinetic investigation

The kinetics of alkaline hydrolysis of efavirenz by 0.1 M sodium hydroxide was investigated. A regular decrease in concentration of efavirenz with increasing time was observed. At the temperatures selected, alkaline hydrolysis followed apparent first order kinetics. From the slopes of the straight lines it was possible to calculate the apparent first order hydrolysis rate constant and half life at each temperature for alkaline hydrolysis of efavirenz (Table 3).

Table 2: Summary of forced degradation study of efavirenz

Stress condition	Time	Assay (%)	Mass balance	Remarks
Acid hydrolysis 48 h	99.90		99.91	No degradation
Base hydrolysis 48 h	65.25		99.98	degradation observed
Oxidation	48 h	99.89	99.90	No degradation
Thermal	7 days	99.91	99.89	No degradation
UV(254 nm)	48 h	78.25	99.88	degradation observed

Table 3: Rate constant (K_{obs}) and half-life ($t_{½}$) for degradation of efavirenz by 0.1 M sodium hydroxide (*NaOH*)

Temperature (°C)	Kobs (h^{-1})	$t_{1/2}$ (h)
30	0.0092	75.32
40	0.011	63.0
50	0.014	49.5
60	0.0178	38.93
70	0.0195	35.5

Table 4: Rate constant (K_{obs}) and half-life ($t_{½}$) for degradation of efavirenz at 30 °C in buffers of different pH.

pH	Kobs (h^{-1})	$t_{1/2}$ (h)
08	0.0004	1732.5
09	0.0010	693.00
10	0.0045	154.00
11	0.0062	111.77
12	0.0072	96.25
13	0.0100	69.30

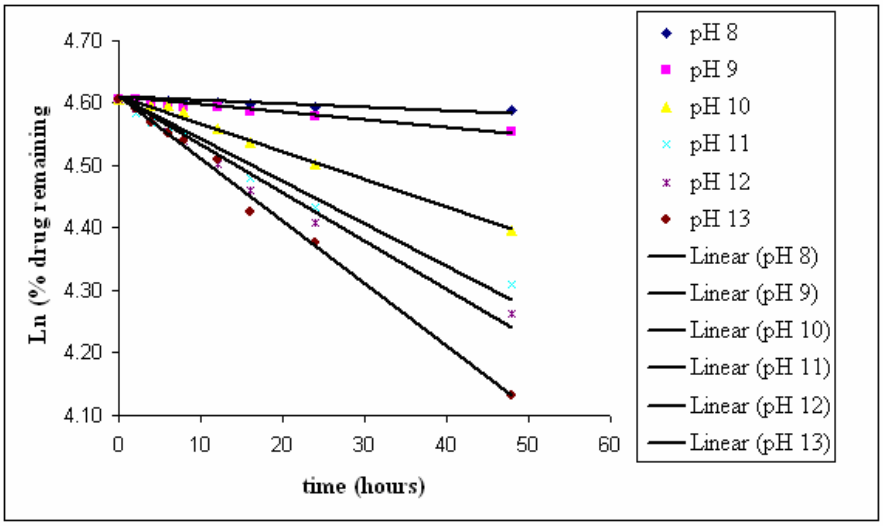

Fig. 4: Apparent first order reaction of efavirenz in aqueous solutions at various pH values

pH Rate Profile

The pH rate profiles were studied at 30 °C (Fig. 4). The degradation rate constant of efavirenz increases in a linear manner as pH increases from 8 to 13(Table 4). The pH of maximum stability is below pH 8.

Validation of Method

Linearity

The linearity of efavirenz standards was evaluated by analyzing a set of standards ranging from 40.0-to 60.0-µg mL^{-1}. The calibration curve parameters of efavirenz showed a linear relationship between peak area and concentration. The mean correlation coefficient, slope and intercept values were 0.9992, 24810, and 5300 respectively.

Precision

The percentage RSD of assay of efavirenz during assay method precision study was within 1 %. The percentage RSD of assay results obtained in intermediate precision study was within 1 % confirming good precision of the method.

Range

The calibration range was established by consideration of the practical range necessary, in accordance with the concentration of efavirenz in pharmaceutical product, to give accurate, precise and linear results.

Limits of Detection and Quantification

In accordance with ICH recommendations [10], the approach based on the standard deviation of the response and the slope of the calibration plot was used for determination of limits of detection and quantification. The limit of detection and quantification of efavirenz was 50 and 160 ng mL^{-1} for 10 µL injection volume respectively.

Accuracy

Percentage recovery of efavirenz in bulk drug samples was ranged from 99.2 to 101.3%. The excellent recovery obtained suggests the accuracy of the method is good.

Robustness

In all the deliberate varied chromatographic conditions (flow rate, percent organic strength, column temperature), the resolution between efavirenz and its hydrolysis product was greater than 2, illustrating the robustness of the method.

Solution Stability and Mobile Phase Stability

The % RSD of assay of efavirenz during solution stability and mobile phase stability experiments was within 1 % RSD No significant change was observed in the content of efavirenz during solution stability and mobile phase experiments. The solution stability and mobile phase stability experiments data confirm that efavirenz sample solutions and mobile phase used during assay were stable for at least 48 h.

CONCLUSION

Comparing with the analytical methods reported previously, the proposed LC-PDA-MS method enables simple, accurate, reproducible and fast quantitative analysis of efavirenz in presence of degradation products. The method has been successfully applied to pH rate profile, stability study, degradation kinetics and identification of degradation products. Efavirenz was found unstable in alkaline media but stable in acidic media. The optimum stability of efavirenz was at pH 1-7.

REFERENCES

1. Rouzes A, Berthoin K, Xuereb F, Djabarouti S, Pellegrin JL, Coupet AC, Augagneur S, Budzinski H, Saux MC, Breith D.Simultaneous analysis of antiretroviral drugs. J Chromatogr B 2004; 813: 209-216.
2. Notari S, Bocedi A, Ippolito G, Narciso P, Pucillo LP, Tossini G, Donnorso RP, Gasparrini F, Ascenzi P. Quantification of 8 HIV-1 protease inhibitors. J Chromatogr B 2006; 831: 258-266.

3. Clercq ED. Bioequivalence and pharmacokinetics of two zidovudine formulations. J Clin Virol 2004; 30: 115-133.
4. Csajka C, Marzolini C, Fattinger K, Decosterd LA, Fellay J, Telenti A, Biollaz J, Buclin T. Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection. Clin Pharmacol Ther 2003; 73: 20-30.
5. Gazzard BG. Central nervous system toxoplasmosis in HIV pathogenesis. HIV Med 2000;1: 11-14.
6. Sailaja A, Kumar K, Ravikumar DVR, Kumar C, Yugandhar NM, Srinubabu G. Development and validation of HPLC method for determination of efavirenz in human plasma. chromatographia 2007; 65: 359-361.
7. Dogan-Topal B, Ozkan SA, Uslu B. Simultaneous determination of efavirenz, valganciclovir and abacavir in human serum by HPLC. Chromatographia 2007; 66 :25-30.
8. Montagomery E, Edmanson A, Cook S C, Hovsepian P K. Development and validation of reverse phase HPLC method for analysis of efavirenz. J harm Biomed Anal 2001; 25: 267-284.
9. Ribeiro J, Campos L, Alves R, Lages G, Pianetti G. Efavirenz related compounds preparation by hydrolysis method. J Pharm Biomed Anal 2007; 43 : 298-303.
10. International conference on harmonization (1993) Stability testing of new drug substances and products. ICH, Geneva.
11. Snyder LR, Kirkland JJ, Glajch JL (1997) Practical HPLC method development, 2nd edn. Wiley, New York, p 317.