International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.3, No.3,pp 1889-1896, July-Sept 2011

Stability Study and Densitometric Determination of Efavirenz in Tablet by Normal Phase Thin Layer Chromatography

Rajendra B. Kakde* and Dipak L. Kale

Department of Pharmaceutical Sciences, RTM Nagpur University, Amravati Road, Nagpur, Maharashtra, INDIA-440033

Corres.author: drkakde@yahoo.com,Tel.: +919622711060.

Abstract: A simple, precise and accurate stability indicating normal phase high performance thin layer chromatographic method for determination of efavirenz in tablet preparation has been developed and validated. Efavirenz from the formulations in presence of its degradation product was separated on silica gel 60 F_{254} HPTLC plates with chloroform, methanol and toluene in the proportion of 7:1:2 (v/v) as mobile phase. Densitometric quantification was performed at 252 nm. Well resolved bands were obtained for efavirenz with R_F value 0.70 and for degradation products. The method was validated for specificity, accuracy, precision and robustness. The calibration curve was found to be linear in the concentration range of 500-1000 ng per band both by area and height with correlation coefficients of 0.999 and 0.998 respectively. The method is selective and specific with potential application in pharmaceutical analysis of these drugs in bulk and formulations.
Keywords: Efavirenz, Antiretroviral, Densitometry, Stability, HPTLC, Validation.

Introduction

Efavirenz (EFA) chemically, (4*S*)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoro methyl)-2*H*-3,1-benzoxazin-2-one (Fig. 1), is official in Indian pharmacopoeia[1] and Merck Index[2]. It is non-nucleoside reverse transcriptase inhibitor and is used as part of highly active antiretroviral therapy in the treatment of human immunodeficiency virus type 1[3].

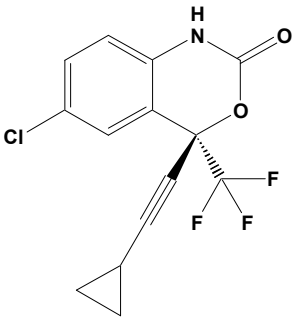

Figure 1. Structural formula of EFA

Literature survey reveals variety of methods like HPLC[4] in capsule, capillary electrophoresis [5], micellor electrokinetic chromatography[6], HPLC[7-12] in plasma alone have been reported. EFA is also estimated in combination with other drugs in biological fluids using HPLC[13-29], in presence of metabolites[30-35], after post column derivatization[36], with related substances [37-39], LC-MS[40-49] and GC-MS[50]. Hamrapurkar et.al.[51] tried to estimate EFA using HPTLC but the method did not describe the stability behavior of EFA and also method is not specific in presence of degradation product. In the present study, an attempt has been made to develop simple, rapid, precise and accurate stability indicating high performance thin layer chromatographic method for analysis of EFA in bulk and marketed formulations in the presence of its potential degradation products.

Material and methods

Chemicals, reagents and solutions

The EFA working standards were obtained as a gift sample from Matrix Laboratories Ltd., Hyderabad, India. The tablet formulation containing EFA (200 mg) is available in market by brand name *Efferven*. Chloroform, methanol and toluene were of analytical grade purchased from Qualigens and pre-coated silica gel 60 F_{254} HPTLC plates from Merck, Darmastadt, Germany. All dilutions were performed in standard volumetric flasks. Double distilled water and Whatmann filter paper Grade-I was used throughout the experimental work.

Preparation of standard solution

An accurately weighed 50.0 mg of pure standard EFA was taken in 50.0 mL volumetric flask and dissolved in 20-25 mL of methanol and made up the volume to obtain standard stock solution of 1 mg/ml concentration. Five milliliters of standard stock solution was transferred to a 50 mL volumetric flask and then diluted to volume with methanol to furnish a working standard solution of concentration 100 µg/ml.

Preparation of sample solution

Twenty *Efferven* tablets were weighed and finely powdered. An accurately weighed tablet powder equivalent to 50.0 mg of EFA was transferred to a 50.0 ml volumetric flask. The powder was dissolved in 30 mL methanol and the solution was sonicated for 15 min. The solution was cooled to room temperature and diluted up to the mark with methanol and filtered through Whatmann Grade I filter paper. Five milliliter of clear filtrate was transferred to a 50 ml volumetric flask and diluted to volume with methanol to furnish sample solution of concentration 100 µg/ml.

Instrumentation and chromatographic conditions

Chromatography was performed on 10 x 10 cm pre-coated silica gel 60 F_{254} HPTLC plates. The plates were pre-washed with methanol and dried in an oven at 105°C for 1 hrs before use. Eight microliter of sample was spotted 10 mm away from the edge of the plates by means of a Camag (Muttenz, Switzerland) Linomat IV automatic sample applicator. The plates were developed to a distance of 80 mm in a Camag twin-trough chamber previously saturated with mobile phase vapor 10 min at room temperature (25±2°C). The chromatographic conditions had previously been optimized to achieve the best resolution and peak shape. Plates were evaluated by densitometrically at 252 nm with a Camag TLC Scanner III, in reflectance-absorbance mode controlled by winCATS software (Version 1.4.1; Camag). The slit dimention was 3.00 mm x 0.45 mm and emitting continuous UV radiation between 190 and 360 nm. The amount of the compound chromatographed was determined from the intensity of diffused reflected light. The typical chromatogram and absorption spectra of tablet formulation is shown in Figure 2.

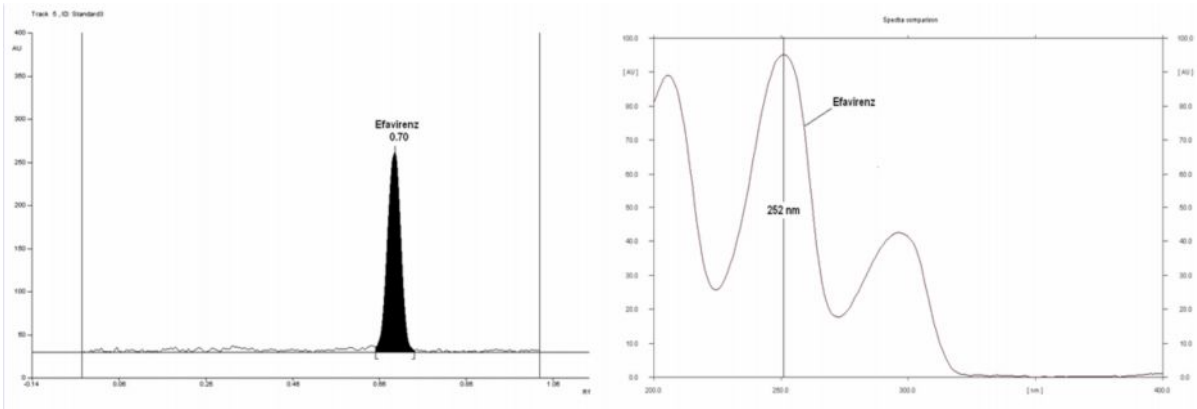

Figure 2. Typical densitograms and in situ spectrum of Efferven tablet

Force degradation studies of EFA

The stress studies were initiated by using 1 mg/ml solution of EFA (API) and exposing it to various stress conditions to study the effect over wide range of pH, heat, oxidation and photo degradation using the following approaches.

Hydrolytic degradation under acidic and alkaline condition was carried by transferring 50 mg of EFA in each of two round bottom flasks and dissolving in 50 ml of 0.1N methanolic hydrochloric acid (methanolic: 50% v/v methanol in distilled water) and 0.1N methanolic sodium hydroxide respectively followed by refluxing on water bath for 8 hrs at 70ºC. Hydrolytic degradation under neutral condition was carried by transferring 50 mg of EFA in 50 ml methanolic water in round bottom flask separately and was refluxed on water bath maintained at 70ºC for 8 hrs. For oxidative degradation, EFA (50.0 mg) was dissolved in 50.0 ml 3% methanolic H_2O_2 in volumetric flask and it was kept in dark place at R.T. for 7 days. Photolytic degradation was carried by evenly spreading EFA in thin layer in a covered petri dish and exposing in sunlight. Thermolytic degradation was carried by transferring EFA in covered petri dish kept in an oven maintained at a temperature of 70ºC.

Similarly, the various degradation products of *Efferven* were prepared by exposing *Efferven* powder equivalent to respective amount of EFA and treating in respective manner.

The samples which showed no degradation at the initial stress conditions were subjected to increasing severity of stress conditions till a certain maximum limit was reached. The limiting maximum stress conditions were assumed on the basis of the available regulatory guidelines, the current pharmaceutical stress testing trends and/or practical limitations imposed by the physicochemical properties of the molecule.

Sampling of Force Degradation Products

Five milliliter of degradation samples (1mg/ml) were withdrawn periodically during hydrolysis under acidic, alkaline and neutral conditions at every, 30 min., 1st, 3rd, 5th and 8th hrs. In case of oxidative stress condition, 5.0 ml of samples were withdrawn every 24hrs for 1st to 7st days. The degradation samples under acidic and basic stress were neutralized with methanolic NaOH or HCl. All stability samples withdrawn were diluted to 10.0 ml with methanol (conc. 0.5 mg/ml). All the samples so prepared were stored under refrigeration. Samples of hydrolytic and oxidative stress studies were further diluted with methanol to get the conc. 100 µg/ml. In case of thermal and photo degradation studies, 10 mg samples were withdrawn every 1st, 3rd, 7th, 15th and 30th day, dissolved in 10 ml of methanol to obtain a resultant concentration of 1 mg/ml each

Preparation of Degradation Sample for HPTLC

The stock degradation samples were diluted suitably with methanol to get concentrations of 100 µg/ml.

Preparation of calibration curve

Aliquot portions of working standard solution (3-14 µl) were applied on the TLC plate and densitograms were developed under optimized chromatographic conditions and the calibration curve was obtained. The curves (Figure 3) were found to be linear between concentration range 500-1000 ng/spot both by height and area. Results have been shown along with correlation coefficient, slope and y-intercept in Table 1.

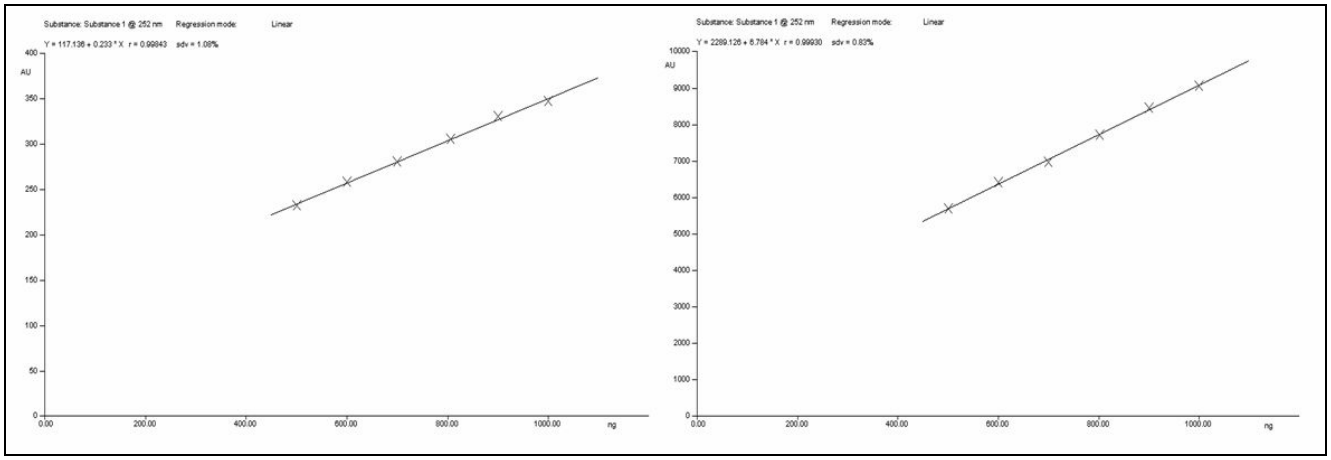

Figure 3. Linearity by height and area for EFA

Table 1. Analytical Performance Data

Parameters	By height	By area
Linear dynamic range (ng/band)	500–1000	500–1000
Slope	0.233	6.784
Y-intercept	117.136	2289.126
Correlation coefficient (r)	0.998	0.999
% Standard Deviation	1.08	0.83
LOD (µg/ml)	164.16	138.45
LOQ (µg/ml)	497.45	419.55

Application of Proposed Method for Estimation in Marketed Formulations

Two bands of working standard and six bands of sample solution of equal volume (8 µL) were applied on TLC plate and the plate was developed and scanned as per optimized chromatographic conditions.

Validation of Proposed Method

The proposed method was validated for specificity, linearity & range, accuracy, precision, limit of detection & limit of quantitation, robustness and ruggedness. Validation of the proposed method was carried in accordance with the ICH guidelines[52,53].

Specificity

The specificity of the method was ascertained by how accurately and specifically the analyte of interest are estimated in the presence of other components (e.g. impurities degradation products etc) by exposing the sample to different stress conditions such as acidic (0.1 N HCl), alkaline (0.1N NaOH), oxidizing (3% H_2O_2), heat (60°C) and UV radiations for 24 hrs and then analyzing them by proposed method.

Accurately weighed quantities of tablet powder equivalent to about 50 mg of EFA (134.46 mg) were transferred to six different 50.0 ml volumetric flasks. The samples were then exposed to stress conditions for 24 hrs. After 24 hrs the flasks were cooled to room temperature, sonicated with 30 ml methanol for 15 min and volumes were made up to 50.0 ml with methanol and filtered through Whatmann Grade I filter paper. Five milliliters of filtrate was transferred to a 50 ml volumetric flask and then volume was made up to the mark with methanol to obtain resultant sample solutions of concentration 100 µg/ml. The solutions were then analyzed in similar manner as described under estimation of marketed formulation.

Linearity and Range

Aliquot portions of working sample solution (3-14 µl) were applied on the TLC plate and densitograms were developed under optimized chromatographic conditions. The graph was plotted as concentration Vs response (peak area or height).

Accuracy

The accuracy of the experiment was established by spiking preanalyzed sample with known amounts of the corresponding drugs at three different concentration levels *i.e.* 80, 100 and 120% of the drug in the tablet (the external standard addition technique). The spiked samples were then analyzed for five times at each level.

Precision

Two bands of working standard solution and five bands of sample solutions of equal volume (8 µL) were spotted on the plate and the plate was developed and evaluated as described above. The procedure was repeated five times, individually weighing the tablet powder each time. The densitometric responses from the standard and sample were used to calculate the amounts of the drug in the tablet. The procedure was repeated five times, individually weighing the tablet powder each time.

Limit of detection and limit of quantitation

LOD and LOQ determination were done with method based on standard deviation of the response and the slope of calibration curve.

Robustness

Robustness was checked by analysis of sample solutions after making small changes to method parameters like change in determination wavelength, working temperature and saturation time.

System suitability test

A system-suitability experiment was performed before determination of EFA in unknown samples.

Results

EFA from the formulations were separated with chloroform, methanol and toluene in the proportion of 7:1:2 (v/v) as mobile phase. Well resolved peak of EFA obtained at R_F value 0.70 at 252 nm as shown in Figure 2.

Forced Degradation study of API and Tablet

Results of degradation products under various conditions are shown in Fig. 4. Results of acidic degradation showed that one degradation product was formed on refluxing in 2N HCl for 5 hrs and on refluxing with 0.1N NaOH for 1 h. EFA was stable under neutral pH for at least 8 hrs refluxing at 70ºC. Oxidative degradation shows two degradation products when EFA exposed to 3% H_2O_2 for 7 days. EFA is found to be stable at thermal degradation at 70°C for 30 days whereas photo degradation was occurred on 15 days in sunlight exposure with two degradation products. The results of degradations in both the pure API and the marketed preparation were identical.

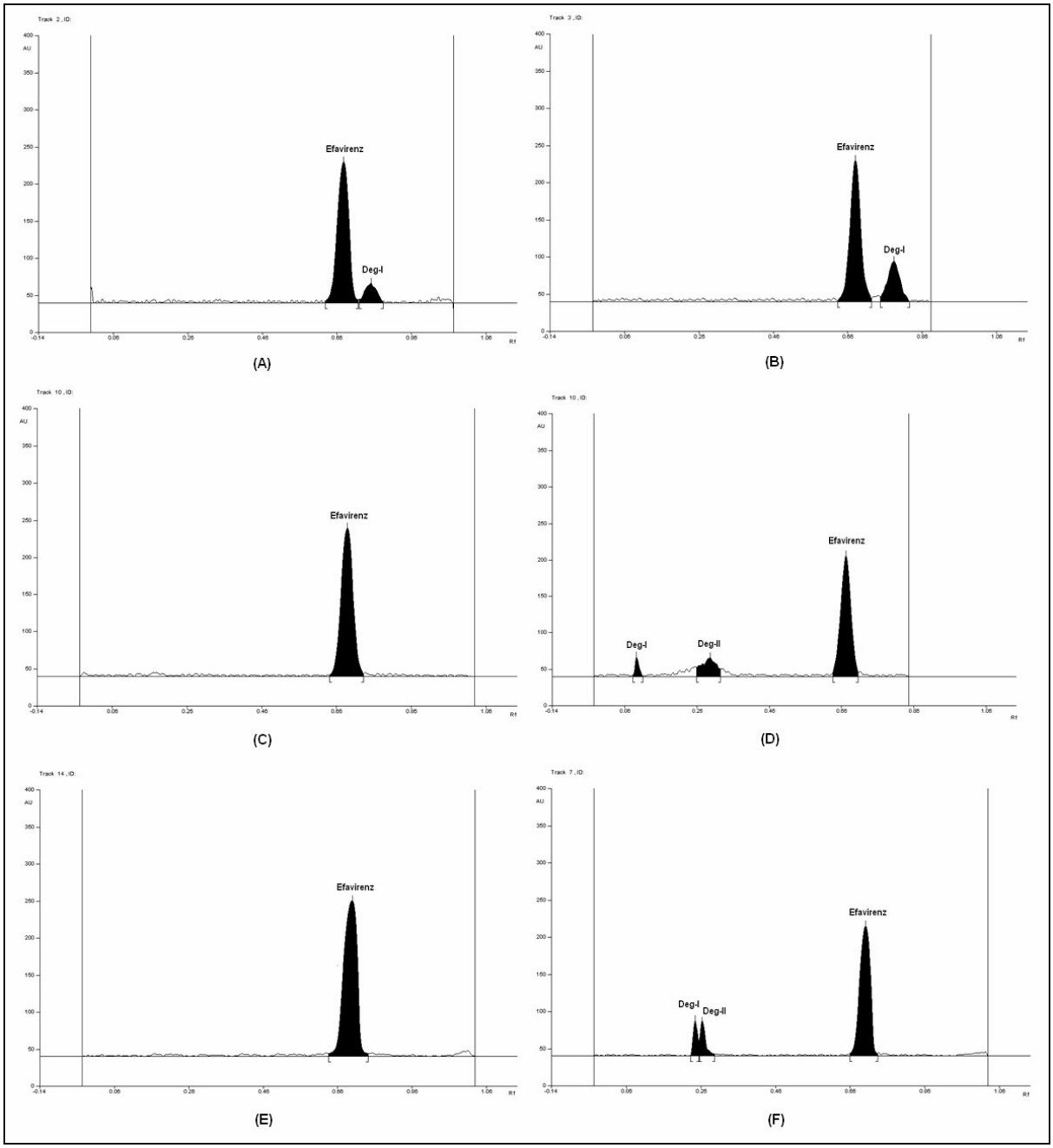

Figure 4. HPTLC densitograms of forced degraded samples of EFA-

(A) 2N HCl reflux for 5 hrs, (B) 0.1N NaOH reflux for 1hrs, (C) Neutral reflux for 8hrs, (D) Treated with 3% H_2O_2 for 7days, (E) Thermal (dry heat) for 30 days at 70° and (F) Sunlight for 15 days

Validation

Specificity study shows that EFA can be estimated using given solvent system without any interference of the possible degradation products and impurities. Calibration curve was found to be linear in the concentration range of 500-1000 ng per band by height and area with correlation coefficients of 0.998 and 0.999 respectively. The linear regression equations were found to be Y = 0.233X + 117.136 and Y = 6.784X + 2289.126 by height and area respectively. Linearity curves are shown in Figure 3. The statistical data obtained are given in Table 1. Mean recovery by the proposed method was within acceptable limits, indicates the method is accurate. The results of recovery study were given in Table 2.

For Precision study, the densitometric responses from the standard and sample were used to calculate the amounts of the drug in the tablet. Percentage estimation was near to 100% with RSD below 2% indicate the method is precise. The results of system, method and intermediate precision studies are shown in Table 3.

Table 2. Results from recovery analysis

Efferven Tablet *(Avg. Wt. 537.82 mg for 200 mg of EFA)*						
Sr. No.	**% Spiking Level**	**Wt. of sample + std. EFA[#] (mg)**	**Amt. of std. recovered (mg)**		**% Recovery***	
			By Height	**By Area**	**By Height**	**By Area**
1	80	94.54 + 5.0	5.02	5.11	100.40	102.10
2	100	94.63 + 15.0	14.75	14.90	98.33	99.32
3	120	93.94 + 25.0	24.82	24.81	99.29	99.24
				Mean	99.34	100.22
				SD	1.0343	1.6295
				% RSD	1.0411	1.6260

*Each value is a mean of five determinations, [#]Added in the form of standard stock solution
SD = Standard deviation, RSD = Relative standard deviation

Table 3. System, method and intermediate precision data

Formulation	**Parameter**		**System Precision**	**Method Precision**	**Intermediate Precision**		
					Interday	**Intraday**	**Different Analysts**
Efferven	Height	Mean*	98.89	98.89	98.43	99.32	98.65
		SD	0.5640	0.5500	1.0836	0.8004	0.9287
		% RSD	0.5703	0.5562	1.1009	0.8059	0.9414
	Area	Mean*	99.12	99.23	99.48	99.79	98.61
		SD	0.3914	0.5832	0.7050	0.6043	0.9605
		% RSD	0.3949	0.5877	0.7088	0.6055	0.9741

*Each value is a mean of five determinations
SD = Standard deviation, RSD = Relative standard deviation

LOD were 164.16, 138.45 and LOQ were 497.45, 419.55 by height and area respectively. The low value of %RSD shows the method is robust and slight change in estimation wavelength and concentration of toluene does not vary the results.

Discussion

Degradation study of pure EFA and formulation is quite important for hydrolysis under acidic and basic conditions and also for oxidative and photo degradation as EFA get degradaed upto the predictable degradation under all these conditions. Acidic degradation upto 11% and basic degradation unto 20% of standard drug are useful to find out possible degradation pathway. No degradation in neutral stress condition suggests that the drug can be kept at condition of neutral pH and confirms stability. EFA is found to be very prone to oxidation and nearly 19% degraded under sunlight over 15 days whereas it is quite stable over 7 days of photo exposure. Thermal degradation at 70°C upto 30 days did not show any degradation confirms practical stability of EFA under varying temperature.

In conclusion, the method is simple, precise and accurate for the determination of EFA and its degradation product in bulk drug and pharmaceutical preparations. Method was validated for precision, accuracy, specificity and robustness and can therefore be applied for routine quality control analysis of EFA in pharmaceutical preparation.

Acknowledgement

The authors extend their sincere thanks to Matrix Laboratories Ltd., Hyderabad, India for providing gift sample of pure efavirenz. We also extend our thanks to Head of Department, Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University for providing the necessary facilities.

References

1. Government of India Ministry of Health and Family Welfare, Indian Pharmacopoeia, The Indian Pharmacopoeia Commission, Ghaziabad, 5th ed., 2007, 1071.
2. O'Neil, M.J., Budawari, S., The Merck Index, Merck and Co Inc, New Jersey, 14th ed., 2006, 598.
3. Keiser, P., Nassar, N., Expert Opin. Pharmacother, 2007, 4, 477-478.
4. Montgomery, E.R., Edmanson, A.L., Cook, S.C., Hovsepian, P.K., J. Pharm. Biomed. Analysis, 2001, 25, 267-284.
5. Pereira, E.A., Micke, G.A., Tavares, M.F., J. Chromatogr. A, 2005, 1091, 169-176.
6. Fan B., Stewart T.J., J. Liq. Chrom. Relat. Tech., 2002, 25, 937-947.
7. Villani P., Pregnolato M., Banfo S., Rettani M., Burroni D., Seminari E., Maserati R., Regazzi B.M., Ther. Drug Monit., 1999, 21, 346-350.
8. Veldkamp I.A., Van Heeswijk R.P., Meenhorst L.P., Mulder W.J., Lange M.J., Beijnen H.J., Hoetelmans M.R., J. Chromatogr. B., 1999,734, 55-61.
9. Ramachandran G., Kumar K.A., Swaminathan S., Venkatesan P., Kumaraswami V., Greenblatt J.D., J. Chromatogr. B., 2006, 835, 131-135.
10. Mogatle S., Kanfer I., J. Pharm. Biomed. Anal., 2009, 49, 1308-1312.
11. Raras-Nacenta S.M., Lopz-Pua L.Y., Cortes L.L.F., Mallolas J., Gatell M.J., Carne X., J. Chromatogr. B., 2001, 763, 53-59.
12. Langmann P., Schirmer D., Vath T., Zilly M., Klinker H., J. Chromatogr. B., 2001,755, 151-156.
13. Marzolini C., Telenti A., Buclin T., Biollaz J., Decosterd A.L., J. Chromatogr. B., 2000, 740 43-58.
14. Usami Y., Oki T., Nakai M., Sagisaka M., Kaneda T., Chem. Pharm. Bull., 2003, 51, 715-718.
15. Rezk L.N., D.R. Crutchley, Yeh F.R., Kashuba D.A., Ther. Drug Monit., 2006, 28 , 517-525.
16. Rebiere H., Mazel B., Civade C., Bonnet A.P., J. Chromatogr. B., 2007, 850, 376-383.
17. Aymard G., Legrand M., Trichereau N., Diquet B.. J. Chromatogr. B., 2000, 744, 227-240.
18. Kappelhoff S.B., Rosing H., D.A. Huitema, Beijnen H.J., J. Chromatogr. B.,2003, 792, 353-362.
19. Boffito M., Tija J., Reynolds E.H., Hoggard G.P., Bonora S., Perri Di G., Back J.D.. Ther. Drug Monit., 2002, 24, 670-674.
20. Turner L.M., Walker R.K., King R.J., Acosta P.E., J. Chromatogr. B., 2003, 784, 331-341.
21. Rezk L.N., Tidwell R.R, Kashuba D.A., J. Chromatogr. B., 2002, 774, 79-88.
22. Rezk N.L., Tidwell R.R., Kashuba D.A., J. Chromatogr. B., 2004, 805, 241-247.

23. Notari S., Bocedi A., Ippolito G., Narciso P., Pucillo P.L., Tossini G., Donnorso P.R., Gasparrini F., Ascenzi P., J. Chromatogr. B. ,2006, 831, 258-66.
24. A. D'Avolio, Baietto L., Siccardi M., Sciandra M., Simiele M., Oddone V., Bonora S., Di Perri G., Ther. Drug Monit., 2008, 30, 662-669.
25. Choi O.S., Rezk L.N., Kashuba D.A., J. Pharm. Biomed. Anal., 2007, 43, 1562-1567.
26. Weller R.D., Brundage C.R., Balfour H.H., Vezina E.H., J. Chromatogr. B., 2007, 848, 369-373.
27. Takahashi M., Yoshida M., Oki T., Okumura N., Suzuki T., Kaneda T.. Biol. Pharm. Bull., 2005, 28, 1286-1290.
28. Proust V., Toth K., Hulin A., Taburet M.A., Gimenez F., Singlas E., J. Chromatogr. B., 2000, 742, 453-458.
29. Seshachalam U., Rao N.D., Chandrasekhar B.K., Pharmazie., 63 2008, 107-109.
30. Moal L.G., Venisse N., Faux J., Chromatographia. 2003, 58, 421-426.
31. Hirabayashi Y., Tsuchiya K., Kimura S., Oka S., Biomed. Chromatogr., 2006, 20, 28-36.
32. Poirier M.J., Robidou P., Jaillon P., Ther. Drug Monit., 2002, 24, 302-309.
33. Poirier M.J., Robidou P., Jaillon P., Ther. Drug Monit., 2005, 27, 186-192.
34. Dailly E., Raffi F, Jolliet P., J. Chromatogr. B., 2004, 813, 353-358.
35. Keil K., Frerichs V. A., Francesco D.R., Morse G., Ther. Drug Monit., 2003, 25 , 340-346.
36. Matthews Z.C., Woolf J.E., Mazenko S.R., Haddix-Wiener H., Chavez-Eng M.C., Constanzer L.M., Doss A.G., Matuszewski K. B., J. Pharm. Biomed. Anal., 2002, 28, 925-934.
37. Montgomery R.E., Edmanson L.A., Cook C.S., Hovsepian K.P., J. Pharm. Biomed. Anal., 2001, 25, 267-284.
38. Ribeiro A.J.,Campos M.L., Alves J.R., Lages P.G., Pianetti P.G., J. Pharm. Biomed. Anal., 2007, 43, 298-303.
39. Weissburg P.R., Montgomery R.E., Junnier A.L., Segretario J., Cook S., Hovsepian K.P., J. Pharm. Biomed. Anal., 2002, 28, 45-56.
40. Colombo S., Beguin A., Telenti A., Biollaz J., Buclin T., Rochat B., J. Chromatogr. B. ,2005, 819, 259-276.
41. Rentsch. M. K., J. Chromatogr. B., 2003,788, 339-350.
42. Volosov. A., Alexander C., Ting L., Soldin S., Clin. Biochem., 2002, 35, 99-103.
43. D'Avolio A., Simiele M,. Siccardi M,. Baietto L, Sciandra M., Bonora S., Perri Di G., J. Pharm. Biomed. Anal., 2010, 52, 774-780.
44. Nirogi R., Bhyrapuneni G., Kandikere V., Mudigonda K., Komarneni P., Aleti R., Mukkanti K., Biomed. Chromatogr. 2009, 23, 371-381.
45. Wolfgang E.J., Matthias U., Claus N., Muhammad B., Sumiko H., Leslie Z.B., Uwe C., Ther. Drug Monit., 2004, 26, 546-562.
46. Heine.R.T., Alderden-Los C.G., Rosing H., Hillebrand M.J., van Gorp E.C.,. Huitema A.D, Beijnen J.H., Rapid Commun. Mass. Spectrom., 2007, 21, 2505-2514.
47. Koal, T., Burhenne, H., Romling, R., Svoboda, M., Resch, K., Kaever, V., Rapid Commun. Mass Spectrom, 2005, 19, 2995-3001.
48. Rouzes, A., Berthoin, K., Xuereb, F., Djabarouti, S., Pellegrin, I., Pellegrin, J.L., Coupet A.C., Augagneur, S., Budzinski, H., Saux, M.C., Breilh, D., J. Chromatogr. B., 2004, 813, 209-216.
49. Fan, B., Bartlett, M.G., Stewart, J.T., Biomed. Chromatogr., 2002, 16, 383-389.
50. Lemmer, P., Schneider, S., Schuman, M., Omes, C., Arendt, V., Tayari, J.C., Fundira, L., Wennig, R., Ther. Drug Monit, 2005, 27, 521-525.
51. Hamrapurkar, P., Phale, M., Shah, N., J. Young Pharmacist, 2009, 1, 359-363.
52. PART II: Validation of analytical procedure: methodology Q2B, ICH Harmonized Tripartite Guidelines, 1996.
53. International Conference on Harmonization, Validation of Analytical Procedures: Methodology, Federal Register, 1996.

*****