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Statistical Comparison of Two Liquid Chromatographic Methods for Ritonavir in Tablets

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Two high performance liquid chromatography (HPLC) methods were developed for quantitation of ritonavir in raw material and tablet dosage form. The methods were validated and statistically compared. Both methods are carried out in a C_{18} column using the PDA detector at 240 nm. The first method, method A is carried out using methanol: acetonitrile: water in the ratio 87:10:3 (v/v/v) as mobile phase at a flow rate of 1 mL/min. The second method, method B is carried out using acetonitrile:methanol:buffer (pH = 6.2 ± 0.05) in the ratio 60:20:20 (v/v/v) as mobile phase at 1 mL/min. Both methods were validated according to ICH guidelines, showed good accuracy, precision, linearity and sensitivity. Two way anova and student's *t*-test was used to correlate the two methods and applied to raw materials and tablet dosage form. There was no significant difference between the two methods.

Key Words: Ritonavir, HPLC methods, Validation, Two way ANOVA, Student's *t*-test.

INTRODUCTION

Ritonavir (RTV) is a selective, competitive and reversible inhibitor of the human immunodeficiency virus (HIV) protease enzyme. Chemically it is (5S, 8S, 10S, 11S)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-*bis*(phenyl methyl)-2,4,7,12-tetraazatridecan-13-oic acid 5-thiazolyl methyl ester (**1**) (Fig. 1). Ritonavir is official in IP[1], BP[2] and USP[3]. Ritonavir selectively inhibits liver enzyme cytochrome P450 (CYP3A) which helps in increase in bioavailability of other Protease inhibitors like Atazanavir sulphate or Lopinavir in dual protease therapy[4]. From the literature review, it was found that ritonavir has been analyzed by HPLC in biological samples and pharmaceutical dosage form[5-15] singly or in combination with other antiretroviral drugs. Sudha *et al.*[16] developed an HPTLC method in single dosage form while Sulebhavikar *et al.*[17] developed an HPTLC method in combined dosage form with lopinavir. Garren *et al.*[18] studied the bioavailability of generic ritonavir and lopinavir tablet in dog model. Rao *et al.*[19] reported stress degradation study by LC/MS method.

The chromatographic methods used for quantitation of ritonavir in biological samples and pharmaceutical dosage form involved, use of tedious sample preparation. The official methods in IP or BP involve use of solvent system of specific pH, use of peak reagent like sodium hexane sulfonate and thermal regulation of the columns (45 and 60 ºC, respectively). Heine *et al.*[8] reported a gradient method in which the ritonavir got separated at 34 min. Chiranjeevi *et al.*[12] also reported an isocratic method in which potassium dihydrogen orthophosphate was used for preparation of buffer and the pH was adjusted by orthophosphoric acid to 4.0. The mobile phase consisted acetonitrile: buffer in the ratio 50:50 (v/v), retention time found at 5.1 min in a 100 mm column length. This inspired the authors to develop simple cost effective isocratic methods with organic solvents and with buffer system to separate ritonavir in raw material or dosage form. These methods are applied to two different brands of ritonavir and validated according to International Conference on Harmonization (ICH) guidelines[20]. Two way Annova and Student's *t*-test was used to correlate the two methods and applied to raw materials and tablet dosage form.

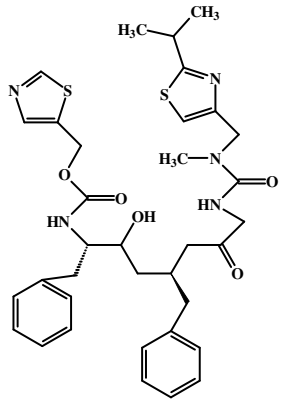

Fig. 1. Chemical structure of ritonavir (RTV)

EXPERIMENTAL

Working standard and API of ritonavir were procured from Matrix Laboratories (Hyderabad, India) as gift sample. Solvents used in both methods and the reagents required for buffer system were of HPLC grade and procured from Merck (Mumbai, India). Two brands of tablets (Ritomune, Cipla and Viriton, Ranbaxy; 100 mg) were purchased from local market.

The chromatographic system consisted of a JASCO (Japan) chromatograph equipped with an LC-Net II/ADC, an MU-2010 Plus PDA Detector, a PU-2089 Plus quaternary pump, an online degasser and a rheodyne model 7725 injector valve with 20 µL sample loop. The chromatograph is coupled with "Chrompass" software (version 1.7.403.1). Separation of ritonavir was done on a HiQSil C18HS (250 mm × 4.6 mm, particle size 5 µm, KYATECH, Japan) under reverse phase partition chromatographic conditions.

For **method A**, mixture of methanol:acetonitrile:water (87:10:3, v/v/v) was used as the mobile phase. All the solvents were filtered through 0.45 µm nylon filter membrane and ultrasonicated before use. The flow rate was 1 mL/min and the assay run time was 10 min. Absorbance was measured at 240 nm.

For **method B**, mixture of acetonitrile:methanol:buffer (60:20:20, v/v/v) was used as mobile phase. The buffer was prepared by adding 2 mL of triethylamine in 1000 mL of water and the pH was adjusted to 6.2 ± 0.05 by orthophosphoric acid. The flow rate was 1 mL/min and the assay run time was 10 min. Absorbance was measured at 240 nm.

Preparation of stock solution, working solution and standard calibration curve: Working stock solution of 1000 µg/mL of ritonavir was prepared by dissolving 25 mg of working standard in 25 mL of methanol. The stock solution was diluted further with methanol to obtain working dilutions of concentrations 200, 150, 100, 50 and 25 µg/mL for **method A** and 400, 300, 200, 100 and 50 µg/mL for **method B**. The prepared samples were also filtered through 0.45 µm membrane filter before injection. The injection volume was 10 µL. The standard calibration curves for both the methods were plotted by AUC *versus* concentration at 240 nm.

Sample preparation: Commercially marketed tablets of ritonavir, ritomune (100 mg, Cipla) and viriton (100 mg, Ranbaxy) were purchased. The samples were prepared by extraction with methanol. Twenty tablets were weighed carefully, average weight was calculated and equivalent amount of solid content was weighed accurately. Weighed amount was dissolved in methanol to prepare the sample solution of concentration 1000 µg/mL. Desired dilution of sample solution was prepared. The sample was filtered through Whatman filter paper No 41, then through 0.45 µm membrane filter before injection.

Method validation: Both the methods were validated according to ICH guidelines[20] for validation of analytical procedure. All the stock solutions and dilutions were prepared in methanol (HPLC grade). The methods were validated in terms of linearity, accuracy and precision, limit of detection (LOD) and limit of quantification (LOQ), robustness and specificity.

Linearity: For linearity of the methods five serial dilutions were prepared from the standard solution. For **method A** the concentration range was 25-200 µg/mL and for **method B** the concentration range was 50-400 µg/mL. Each concentration was injected in triplicate. The linearity was determined by plotting the standard calibration curve by area under curve *versus* concentration. The correlation coefficient was calculated by six point least square regression method.

Accuracy: Accuracy was determined by recovery study by standard addition method. The standard was added to a preanalyzed sample at a concentration level of 25, 50 and 100 %. For method A, 25, 50 and 100 µg/mL of standard solution was added to preanalyzed sample of 100 µg/mL. For method B, 50, 100 and 200 µg/mL of standard solution was added to preanalyzed sample of 200 µg/mL. Each solution was injected in triplicate and recovery was calculated by comparing the peak area with the peak area of standard solution of same concentration.

Precision: Precision of the method was assessed in terms of intra-day and inter-day assay of the sample. System repeatability was determined by six times measurement of a concentration of a sample solution. The concentration of active substance was expressed in terms of relative standard deviation (RSD %) and standard error of mean (SEM). The repeatability of sample assay on the same day accounts for intra-day precision. Inter-day precision was assessed by assay of a concentration for six different days. For **method A**, assay of ritonavir was carried out at 100 µg/mL and for **method B**, assay of ritonavir was carried out at 200 µg/mL, respectively.

Limit of detection and limit of quantification: Limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated. Limit of quantification (LOQ) is the lowest amount of analyte that can be detected in a sample with accuracy and precision. In both the methods, LOD and LOQ was determined using the following equation

$$LOD = \frac{3.3\sigma}{S} \quad (1)$$

$$LOQ = \frac{10\sigma}{S} \quad (2)$$

where 'σ' is the standard deviation of y-intercept and 'S' is the slope of calibration curve.

Robustness: To evaluate method robustness few parameters were varied. The variation was done in composition of solvent system (± 2 % of organic phase), flow rate (± 0.2 mL/min), wavelength (± 2 nm). Robustness was done in triplicate at a concentration level of 100 and 200 µg/mL for ritonavir in method A and B, respectively and the SD of retention time, capacity factor and tailing factor were calculated.

Specificity: To evaluate the specificity of the methods (A and B), two brands of ritonavir tablet were selected, injected and the effect of excipients were studied in respect to retention time, capacity factor, tailing factor and no. of theoretical plates.

Statistical analysis: To correlate the difference between the two developed methods of HPLC, six different samples were taken from two different brands and quantification was done simultaneously. To test difference between the proposed HPLC methods statistical tests were performed for the level of confidence 95 % ($p = 0.05$). Two way ANOVA and Student's

t-test were applied to test the significant difference between both the methods.

RESULTS AND DISCUSSION

Optimization of HPLC methods: Individual methods were developed for ritonavir. As the official methods[1,2] are so much tedious, containing many specific chromatographic conditions of maintenance of specific buffer pH, temperature of column [at 45 ºC (1) or 60 ºC (2)], use of peak reagent (sodium hexane sulfonate), high retention time of 34 min with gradient elution[8] inspired to develop a simple isocratic RP-LC method for the estimation of ritonavir at a faster rate and in a cost effective way. So the **method A** was developed by trial of various solvent systems containing methanol, acetonitrile and water. Finally a solvent system with composition of methanol:acetonitrile:water in the ratio 87:10:3 (v/v/v) at a flow rate of 1 mL/min was found to be the most suitable for quantification of ritonavir at 240 nm. The retention time was found at 3.6 ± 0.04 min (Fig. 2). From literature review[5,7,8,10,21-23], it was found that either too acidic or basic pH were chosen as buffer system for separation of ritonavir in biological samples, so a new method was developed at a pH which can not only be utilized for separation of ritonavir in biological samples but also can prolong the life and performance of the column, a buffer system was developed with triethylamine and ortho-phosphoric acid having pH of 6.2 ± 0.05. The mobile phase developed with buffer system was acetonitrile:methanol:buffer in the ratio 60:20:20 (v/v/v) at a flow rate of 1 mL/min at 240 nm. The retention time of ritonavir was found at 5.547 ± 0.02 min (Fig. 3). Both the methods showed the separation of ritonavir at very early time than the previously reported methods and official methods[1,2].

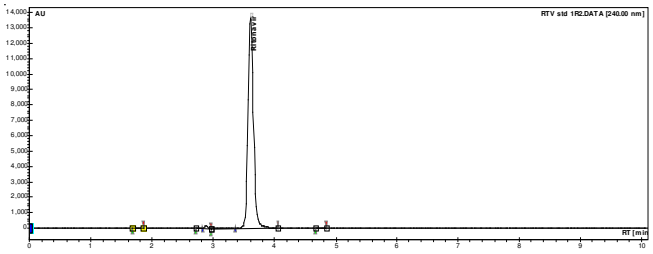

Fig. 2. Representative chromatogram of ritonavir in methanol:acetonitrile: water (87:10:3, v/v/v) at 240 nm

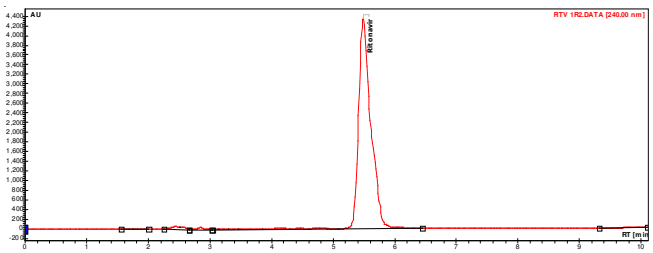

Fig. 3. Representative chromatogram of ritonavir in methanol:acetonitrile: buffer (60:20:20, v/v/v) at 240 nm

Chiranjeevi *et al.*[12] proposed an isocratic method using acetonitrile and buffer in the ratio 50:50 (v/v), retention time was found at 5.1 min, but preparation of buffer with sodium dihydrogen orthophosphate is more tedious than with triethylamine. The column life is more if the pH of the mobile phase is near to 7; more acidic or basic is the pH of the mobile phase, lesser is the column life. To develop methods in which low or high pH mobile phase, require highly specified modified bonded phase stationary phases, but in our method the pH is maintained at 6.2 ± 0.05, so it is easier for maintenance of column in good condition for long period. The mobile phases developed in both methods can be universally applied to any kind of C18 column for separation of ritonavir in raw material and in dosage form. The small laboratories can utilise any one method not only for separation of ritonavir in bulk or dosage form but also can use the developed methods for routine quality assessment and stability study.

Linearity: ritonavir showed good correlation coefficient in concentration range of 25-200 µg/mL (r = 0.9997) and 50-400 µg/mL (r = 0.9995) in method A and B, respectively. Linearity was calculated by determining five standard working solutions containing ritonavir in triplicate for method A and B (Table-1). For both the methods linearity of calibration graphs was validated by high value of correlation of coefficient and the SD for intercept value was less than 2 %. No significant difference was observed in the slopes of standard curves.

TABLE-1
LINEAR REGRESSION DATA FOR CALIBRATION CURVES

Parameters	**Method A**	**Method B**
Linearity range (µg/mL)	25-200	50-400
r ± SD	0.9997 ± 0.0003	0.9995 ± 0.0002
Slope ± SD	612.75 ± 1.23	326.68 ± 1.50
Intercept ± SD	625.05 ± 3.62	699.2 ± 2.78

Accuracy: Both the proposed methods when used for extraction and subsequent quantification of ritonavir in preanalyzed sample by standard addition method at the level of 25, 50 and 100 %. In both the methods assay of each concentration were repeated for three times. The mean recoveries for ritonavir from the marketed formulation are listed in Table-2.

Precision: The intra-day and inter-day precision were determined by assaying the tablets for six times at a concentration level in a day and for consecutive six days and expressed as relative standard deviation. The relative standard deviations were below 2 %, which signifies the precision of both the methods (Tables 3 and 4).

Limit of detection and limit of quantification: The LOD and LOQ were determined for both the methods according to eqns. 1 and 2. For method A, the LOD and LOQ were found to be 0.286 and 0.858 µg/mL, respectively. For method B, the LOD and LOQ were found to be 0.0194 and 0.059 µg/mL, respectively.

Robustness: Robustness of the method was determined by varying in % of organic solvent, change of flow rate and change of wavelength. The factors like retention time, capacity factor and tailing factor were determined and expressed in terms of standard deviation. Thus replicate injections (n = 3) of standard solution was analyzed under slight chromatographic changes (Table-5).

Specificity: The specificity of the HPLC methods was found in complete separation ritonavir in tablets in presence of excipients. The average retention time ± standard deviation

TABLE-2
RECOVERY STUDY BY **METHOD A** AND **B**

Name of the formulation	Method A					Method B				
	Conc. of sample (µg)	Conc. of standard added (µg)	Recovery (%)*	RSD (%)	SEM	Conc. of sample (µg)	Conc. of standard added (µg)	Recovery (%)*	RSD (%)	SEM
Ritomune	100	25	99.95	0.53	0.3	200	50	99.57	0.62	0.24
	100	50	99.47	0.38	0.19	200	100	99.86	0.78	0.14
	100	100	100.01	0.29	0.11	200	200	99.68	0.47	0.12
Viriton	100	25	99.93	0.47	0.19	200	50	99.86	0.42	0.26
	100	50	99.75	0.37	0.27	200	100	99.98	0.31	0.15
	100	100	99.44	0.5	0.19	200	200	99.61	0.22	0.12

*Mean of three determinations.

TABLE-3
INTRADAY PRECISION OF RTV BY **METHOD A** AND **B**

Name of the formulation	Label claim (mg)	Method A			Method B		
		Mean* (mg) ± SD	RSD (%)	SEM	Mean* (mg) ± SD	RSD (%)	SEM
Ritomune	100	99.48 ± 0.78	0.26	0.32	99.55 ± 0.97	0.32	0.40
Viriton	100	99.65 ± 0.43	0.43	0.17	99.69 ± 0.70	0.70	0.29

*Mean of six determinations.

TABLE-4
INTERDAY PRECISION OF RTV BY **METHOD A** AND **B**

Name of the formulation	Label claim (mg)	Method A			Method B		
		Mean* (mg) ± SD	RSD (%)	SEM	Mean* (mg) ± SD	RSD (%)	SEM
Ritomune	100	99.40 ± 0.61	0.2	0.25	99.70 ± 0.32	0.11	0.13
Viriton	100	99.65 ± -0.414	0.41	0.17	99.77 ± 0.32	0.32	0.13

*Mean of six determinations.

TABLE-5
ROBUSTNESS (a) EVALUATION OF **METHOD A** AND (b) EVALUATION OF **METHOD B**

(a) Evaluation of **method A** (Concentration of RTV = 100µg/mL)				
Chromatographic changes		Method A		
Factors	Levels	R_t* (min)	K*	A_s*
Methanol:acetonitrile:water	89:08:03	3.45	2.83	1.09
	87:10:03	3.6	3.33	1.01
	85:12:03	3.78	3.2	0.97
Mean ± SD (n = 3)		3.61 ± 0.165	3.12 ± 0.26	1.02 ± 0.061
Change in the flow rate	0.8 mL/min	3.75	3.16	1.11
	1.0 mL/min	3.6	3.33	1.01
	1.2 mL/min	3.16	2.51	0.95
Mean ± SD (n = 3)		3.50 ± 0.31	3.0 ± 0.42	1.02 ± 0.08
Wavelength	238 nm	3.58	2.97	1.01
	240 nm	3.6	3.33	1.01
	242 nm	3.53	2.92	1.03
Mean ± SD (n = 3)		3.57 ± 0.036	3.07 ± 0.22	1.01 ± 0.02
(b) Evaluation of **method B** (Concentration of RTV = 200 µg/mL)				
Acetonitrile:methanol:buffer	62:18:20	4.89	4.43	1.02
	60:20:20	5.54	5.15	0.96
	58:22:20	6.35	6.05	0.92
Mean ± SD (n = 3)		5.59 ± 0.73	5.21 ± 0.81	1.02 ± 0.061
Change in the flow rate	0.8 mL/min	6.76	6.51	1.08
	1.0 mL/min	5.54	5.15	0.96
	1.2 mL/min	4.78	4.31	0.92
Mean ± SD (n = 3)		5.69 ± 0.99	5.32 ± 1.11	1.02 ± 0.08
Wavelength	238 nm	5.32	4.91	0.98
	240 nm	5.54	5.15	0.96
	242 nm	5.35	4.94	1.02
Mean ± SD (n = 3)		5.40 ± 0.12	5.00 ± 0.13	1.01 ± 0.02

*Mean of three determinations; R_t = Retention time; K= Capacity factor; A_s = Asymmetric factor.

for ritonavir were found to be 3.62 ± 0.03 and 5.547 ± 0.05 min in **method A** and **B**, respectively, for three replicates. The peaks were sharp and had clear baseline separation.

Statistical correlation: Two way ANOVA was applied to test both method-sample interaction and differences in method precision. In both the cases **F stat is less than F crit,**

TABLE-6
TWO WAY ANOVA TEST OF RTV BY DETERMINATION IN SIX INDEPENDENT SAMPLES IN DUPLICATE BY **METHOD A** AND **B**

Sample	Method A		Method B	
	Ritomune	Viriton	Ritomune	Viriton
1	99.83	99.52	99.96	99.68
2	98.72	99.8	98.89	99.89
3	100.05	98.55	98.58	99.95
4	99.57	99.45	99.49	100.05
5	99.82	98.87	100.24	99.36
6	99.88	99.39	99.75	98.37

ANOVA: Two-factor with replication

Source of variation	SS	df	MS	Fstat	P-value	F crit
Sample	0.1504	1	0.1504	**0.4833**	0.4949	**4.3512**
Columns	0.024	1	0.024	**0.0773**	0.7838	**4.3512**
Interaction	0.2993	1	0.2993	**0.9617**	0.3384	**4.3512**
Within	6.2236	20	0.3112	–	–	–

F stat < F crit.

signifying the method-sample interaction and the differences between the methods are not significant (Table-6).

To test means a paired student's *t*-test was applied. The test removes any variation between samples. From the student's *t*-test, t stat < t crit was found in both the cases signifying there is no significant difference between the means (Table-7).

TABLE-7
AVERAGE RESULTS OF RITOMUNE (a) AND VIRITON (b) DETERMINATION BY METHOD A AND B AND THEIR CORRELATION BY PAIRED *t*-TEST

Sample	Method A*	Method B*
(a) Ritomune		
1	99.83	99.96
2	98.72	98.89
3	100.05	98.58
4	99.57	99.49
5	99.82	100.24
6	99.88	99.75
Average	99.645	99.485

t-Test: Paired two sample for means

	Variable 1	Variable 2
Mean	99.645	99.485
Variance	0.229	0.4078
Observations	6	6
Pearson Correlation	0.3048	–
Hypothesized mean difference	0	–
df	5	–
t Stat	**0.5839**	–
P(T ⇐ t) one-tail	0.2923	–
t Critical one-tail	2.015	–
P(T ⇐ t) two-tail	0.58464	–
t Critical two-tail	**2.5706**	–

t Stat < t critical

Sample	Method A*	Method B*
(b) Viriton		
1	99.52	99.68
2	99.8	99.89
3	98.55	99.95
4	99.45	100.05
5	98.87	99.36
6	99.39	98.37
Average	99.26	99.55

t-Test: Paired two sample for means

	Variable 1	Variable 2
Mean	99.2633	99.55
Variance	0.2137	0.3942
Observations	6	6
Pearson correlation	-0.035	–
Hypothesized mean difference	0	–
df	5	–
t Stat	**-0.886**	–
P (T ⇐ t) one-tail	0.2081	–
t Critical one-tail	2.015	–
P(T ⇐ t) two-tail	0.4162	–
t Critical two-tail	**2.5706**	–

t Stat < t critical

*Results are presented as mg of label claim of ritonavir in tablet.

Conclusion

The proposed HPLC methods are simple, accurate and reproducible quantitative method for quantification of ritonavir in dosage form. Both the methods are validated according to ICH guidelines and correlated by statistical analysis. From the statistical correlation it can be concluded that both the methods are useful for quantification of ritonavir with accuracy, precision, less time and in cost effective way than the official methods. As duration of analysis and cost of the analysis is less, both the methods are suitable for determination of ritonavir in pharmaceutical formulation.

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REFERENCES

1. Indian Pharmacopoeia, Indian Pharmacopoeia Commission, Ghaziabad, India, Vol. 3, p. 1059 (2007).
2. British Pharmacopoeia, The Stationary Office, London, Volume 1 and 2, p. 5227 (2009).
3. United State Pharmacopoeia 30-NF25, United States Pharmacopoeal Convention. Inc., Twinbrook Parkway, Rockville, USA, p. 3143 (2007).
4. R.S.Yekkala, D. Ashenafi, I. Marien, H. Xin, E. Haghedooren, J. Hoogmartens and E. Adams, *J. Pharm. Biomed. Anal.*, **48**, 1050 (2008).

5. D.R. Weller, R.C. Brundage, H.H. Balfour Jr. and H.E. Vezina, *J. Chromatogr. B*, **848**, 369 (2007).
6. M.L. Turner, K.R. Walker, J.R. King and E.P. Acosta, *J. Chromatogr. B*, **784**, 331 (2003).
7. S. Frappier, D. Breilh, E. Diarte, B. Ba, D. Ducint, J.L. Pellegrin and M.C. Saux, *J. Chromatogr. B*, **714**, 384 (1998).
8. R. Heine, M. Davids, H. Rosing, E.C.M. Gorp, J.W. Mulder, Y.T. Heide, J.H. Beijnen and A.D. Huitema, *J. Chromatogr. B*, **877**, 575 (2009).
9. Y. Huang, M. Gandhi, R.M. Greenblatt, W. Gee, E.T. Lin and N. Messenkoff, *Rapid. Comm. Mass Spectr*., **22**, 3401 (2008).
10. Y.R. Hu, X.Y. Liu, Z.Z. Zhang and H.L. Qiao, *Yaowu Fenxi Zazhi*, **26**, 1434 (2006).
11. J. van der Lugt, J. Lange, A. Avihingsanon, J. Ananworanich, S. Sealoo, D. Burger, M. Gorowara, P. Phanuphak and K. Ruxrungtham, *Antivir. Therap*., **14**, 1001 (2009).
12. K. Chiranjeevi and K.P. Channabasavaraj, *Int. J. Pharm. Sci. Res*., **2**, 596 (2011).
13. I. Ponnilavarasan, A. Rajasekaran, J.G. Dharuman, D. Kalaiyarasi and M. Senthilkumar, *Dig. J. Nanomater. Biostruc*., **5**, 771 (2010).
14. Y. Usami, T. Oki, M. Nakai, M. Sagisaka and T. Kaneda, *Chem. Pharm. Bull*., **51**, 715 (2003).
15. A. Suneetha, S. Kathirvel and G. Ramachandrika, *Int. J. Pharm. Pharm. Sci*., **3**, 49 (2011).
16. T. Sudha, R. Vanitha and V. Ganesan, *Der. Pharm. Chem*., **3**, 127 (2011).
17. A.V. Sulebhavikar, U.D. Pawar, K.V. Mangoankar and N.D. Prabhunavelkar, *E.-J. Chem*., **5**, 706 (2008).
18. K.W. Garren, S. Rahim, K. Marsh and J.B. Morris, *J. Pharm. Sci*., **99**, 626 (2010).
19. R.N. Rao, B. Ramachandra, R.M. Vali and S.S. Raju, *J. Pharm. Biomed. Anal*., **53**, 833 (2010).
20. International Conference on Harmonization Specifications Q2A (R1): Validation of Analytical Procedures: Text and Methodology Test. November 5-10, Chicago. USA (2005).
21. Y. Harumi, K. Hajime, N. Tetsuya and I. Aikichi, *J. Chromatogr. B: Biomed. Sci. Appl*., **755**, 85 (2001).
22. J. Ray, E. Pang and D. Carey, *J. Chromatogr. B*, **775**, 225 (2001).
23. J. Faux, N. Venisse, G. Le Moal, A. Dupuis and S. Bouquet, *Chromatographia*, **58**, 421 (2003).