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RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LOPINAVIR AND RITONAVIR IN COMBINED TABLET DOSAGEFORM AND IN SPIKED HUMAN PLASMA

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Keywords:

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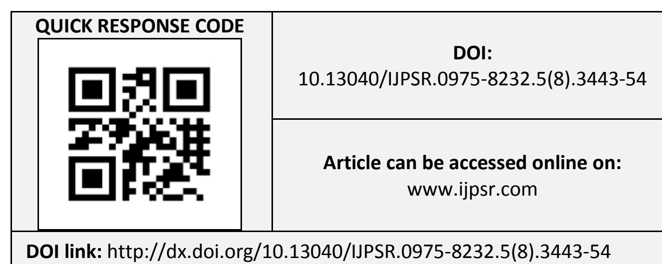
ABSTRACT: A simple, accurate RP-HPLC method has been developed for simultaneous determination of Lopinavir (LPV) and Ritonavir (RTV) from tablet dosage form on reversed phase Brownlee C_{18} column. The sample was analyzed using acetonitrile: 10 mM ammonium acetate buffer (pH 4.5): Methanol (40:30:30) as a mobile phase at flow rate of 1.0 ml/min and detection at 210 nm using UV detector. The limit of detection (LOD) was found to be 0.061 µg /ml and 0.083 µg /ml and the limit of quantification (LOQ) was found to be 0.184 µg /ml and 0.25 µg /ml for LPV and RTV, respectively. Linearity was observed in the concentration range of 5-35 µg/ml. Both the drugs were spiked in 5% proportion with human plasma, extracted by plasma protein precipitation by using acetonitrile and analyzed by HPLC. The retention time for RTV and LPV was found to be 10.30 and 12.58 min respectively. The % recoveries of RTV and LPV were found to be 86.38 to 93.24% and 88.56 to 92.34% respectively. The LOD was found to be 86.80 ng /ml and 67.86 ng /ml as well as the LOQ was found to be 258.47 ng /ml and 193.75 ng /ml for LPV and RTV, respectively.

INTRODUCTION: Lopinavir (LPV) [1S-[1R*,(R*), 3R*, 4R*]]-N-[4-[[(2,6-dimethyl phenoxy) acetyl] amino]-3-hydroxy-5-phenyl- 1-phenyl methyl) pentyl] tetrahydro –alpha - (1-methyl ethyl) -2-oxo-1(2H) –pyrimidine acetamide (**Figure 1A**) and Ritonavir (RTV) [10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methyl ethyl)-4-thiazolyl] -3,6-dioxo -8,11-bis (phenyl methyl)-2,4,7,12-tetraaza tridecan-13-oic acid, 5-thiazolyl methyl ester, [5S-5R*,8R*,10R*,11R*)] (**Figure 1B**) are anti-HIV (HIV protease inhibitors) drugs. In literature, LPV and RTV have been reported to be quantified individually or in combination by spectrophotometric methods [1–3], HPTLC method [4], HPLC methods [5-8] from bulk drug and dosage forms as well as RP-HPLC/MS methods [9–13] for simultaneous determination of LPV and RTV and in combination with other antiviral drugs in the biological matrices which requires very costly instrumentation system for analysis.

So it was aimed to develop and validated simple, sensitive and cost effective RP-HPLC method for simultaneous estimation of LPV and RTV in bulk, their combined tablet dosage form and human plasma.



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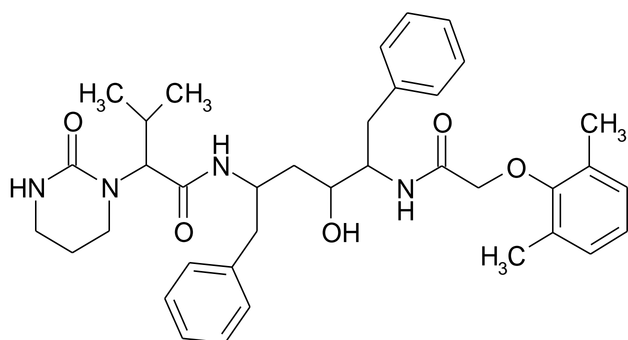


FIGURE 1A: CHEMICAL STRUCTURE OF LOPINAVIR

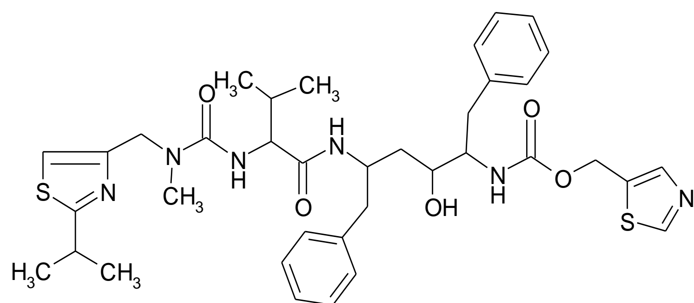


FIGURE 1B: CHEMICAL STRUCTURE OF RITONAVIR

EXPERIMENTAL:

For Bulk and Tablet Dosage Form:

1. **Chemicals and reagents:** LPV and RTV were procured as gift samples from Emcure Pharmaceuticals Ltd., Pune, India. Tablets (Lopimune, Cipla Ltd) containing LPV (200mg) and RTV (50mg) were purchased from the local market. Ammonium acetate (AR), acetonitrile and water HPLC grade (Rankem, RFCL Ltd., New Delhi, India) were used in the study.

2. **Instrumentation**: Perkin Elmer HPLC system (USA) consisted of Perkin Elmer series 200 pump system with Rhenodyne valve injector having 20 µL volume capacity and UV-visible detector. Brownlee C_{18} column (250 mm x 4.6 mm, 5 µm) was used for chromatographic separation.

3. **Mobile phase preparation:** The mobile phase was prepared by mixing 40 mL acetonitrile, 30 mL 10mM ammonium acetate buffer (pH 4.5±0.05 adjusted with orthophosphoric acid) and 30 mL methanol previously filtered through nylon 0.45µm membrane filter. The mobile phase was degassed for 15 minutes before use.

4. **Selection of Wavelength for Determination:** The standard solutions of LPV and RTV were scanned in the range of 200 -400 nm against reagent blank. Both the drugs showed significant absorbance at 210 nm which was selected for analysis.

5. **Standard preparation:** LPV (25 mg) and RTV (25 mg) were accurately weighed and transferred to a 25 ml volumetric flask and dissolved in 15ml methanol. The flask was shaken and volume was made up to the mark with methanol to give a solution containing 1000 µg/ml LPV and 1000 µg/ml RTV. Aliquot 0.05ml, 0.1ml, 0.15ml, 0.2ml, 0.25ml, 0.3ml and 0.35ml were transferred to different volumetric flasks of 10 ml capacity. The volume was adjusted to the mark with methanol to give a solution containing 5, 10, 15, 20, 25, 30 and 35 µg/ml LPV and RTV. The mixed standard solutions were used for calibration purpose.

6. **Sample preparation**: Twenty tablets were weighed and finely powdered. The powder equivalent to 10mg LPV and 2.5 mg RTV was accurately weighed and transferred to volumetric flask of 10ml capacity. Methanol (6 ml) was added and sonicated for 20 mins. The flask was shaken and volume was made up to the mark with methanol and filtered through Whatman filter paper (No. 41). Aliquot (1 ml) was transferred to 10 ml volumetric flask and diluted up to the mark with methanol to give a solution containing 100 µg/ml LPV and 25 µg/ml RTV. The solution (2 ml) was transferred to 10 ml vol. flask. Volume was made up to the mark with methanol to give a solution containing 20µg/ml LPV and 5 µg/ml RTV.

7. **Chromatographic conditions:** The chromatographic separation was achieved on Brownlee C_{18} column (250 mm x 4.6 mm, 5 µm). The HPLC system was operated isocratically at 25°C column oven temperature, using mobile phase comprised

of acetonitrile : 10 mM ammonium acetate buffer (pH 4.5 ± 0.05 adjusted with orthphosphoric acid) : Methanol (40:30:30 v/v/v), at a flow rate of 1.0 mL/min. The mobile phase was filtered through nylon 0.45µm membrane filter and was degassed before use. The determination was carried out at 210 nm wavelength by UV-Visible detector. The injection volume was 20 µL and total run time was 15 min.

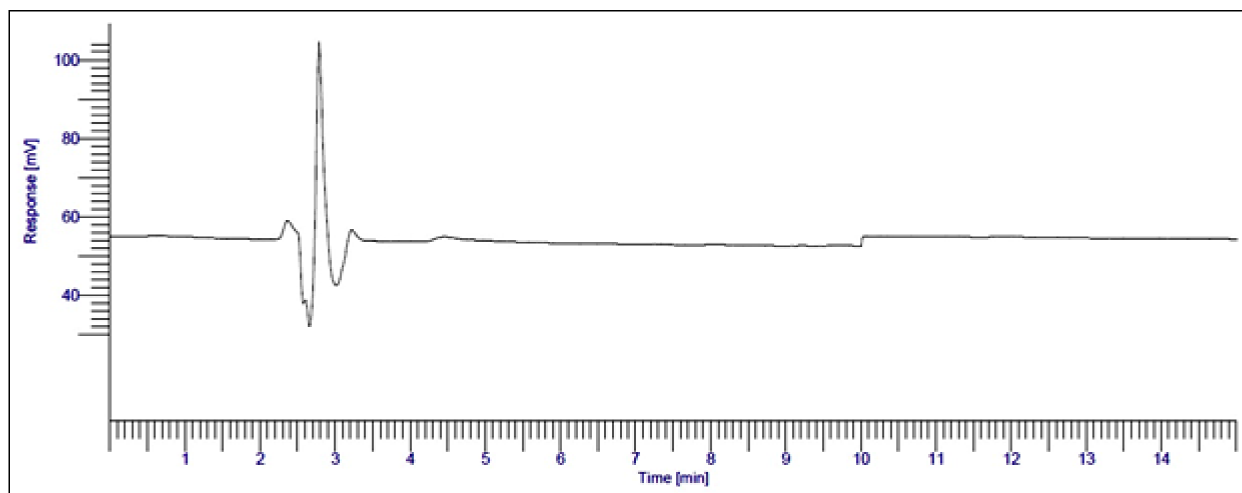


FIGURE 2: HPLC CHROMATOGRAM OF METHANOL BLANK AT 210 NM

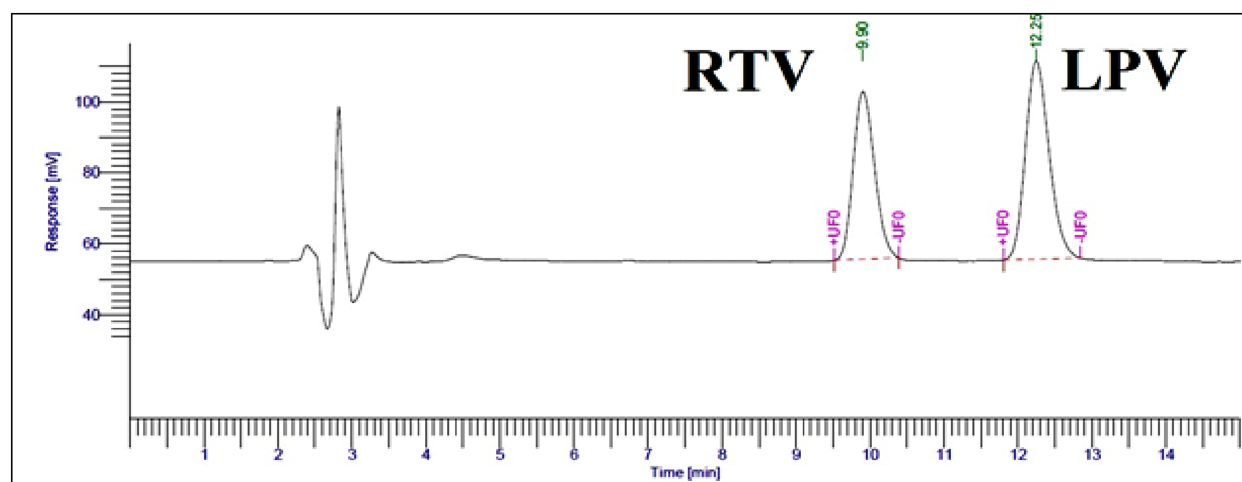


FIGURE 3: HPLC CHROMATOGRAM OF RTV (35 µg/ml) AND LPV (35 µg/ml) STANDARD WITH CORRESPONDING RETENTION TIME AT 210nm

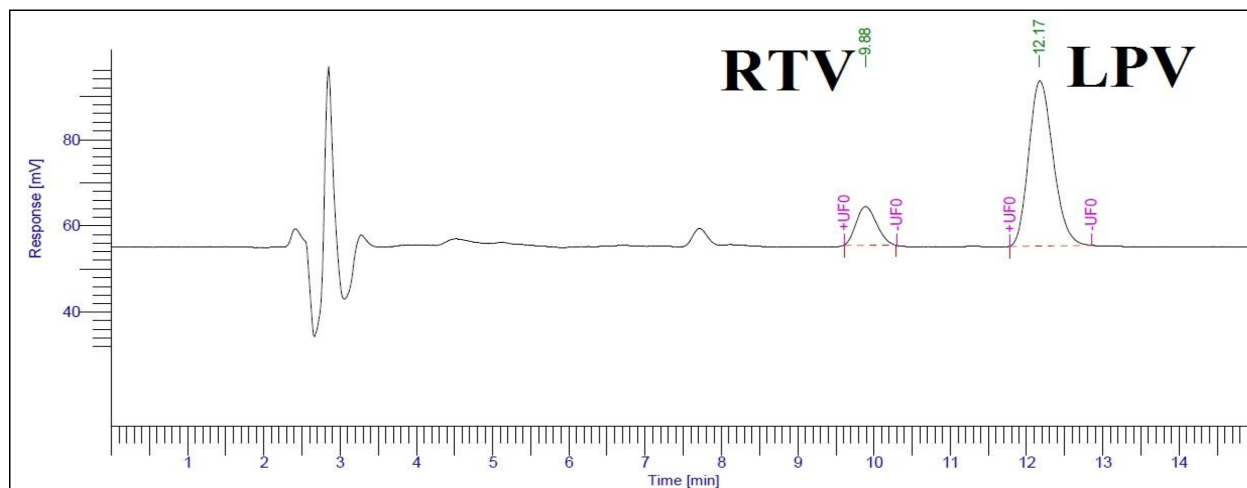


FIGURE 4: HPLC CHROMATOGRAM OF RTV (5 µg/ml) AND LPV (20 µg/ml) TABLET SAMPLE WITH CORRESPONDING RETENTION TIME AT 210nm

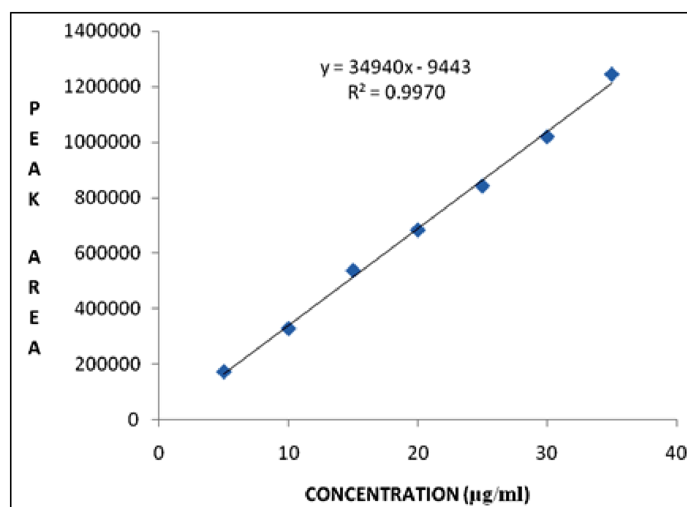


FIGURE 5: CALIBRATION CURVE OF LPV IN BULK

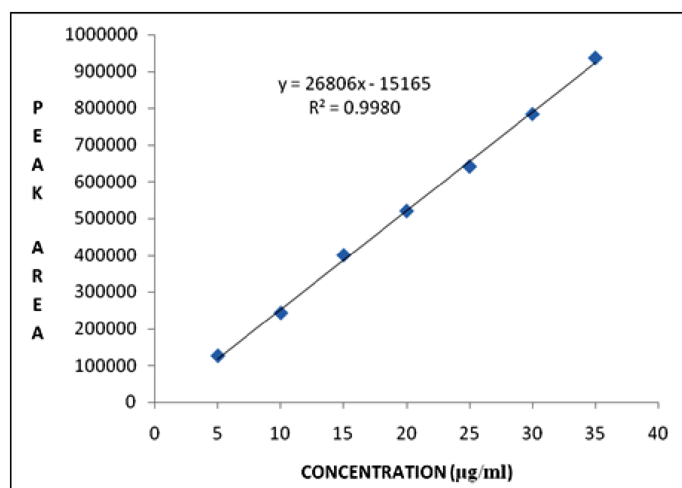


FIGURE 6: CALIBRATION CURVE OF RTV IN BULK

8. **Estimation of LPV and RTV in combined tablet dosage form:** Sample solution was chromatographed for 15 minutes using aforementioned mobile phase at a flow rate of 1.0 ml/min. From the peak area obtained in the chromatogram, the amounts of both the drugs were calculated by the regression equation of the respective drug.

9. **Method validation:** The developed method was validated as per the International Conference on Harmonization (ICH) [14, 15] guidelines with respect to linearity and range, specificity, precision, accuracy, robustness, limit of detection and limit of quantification.

For Spiked human plasma:

1. **Preparation of LPV and RTV standard solutions and quality control samples:** Accurately weighed LPV and RTV (25 mg each) were transferred to same 25 mL volumetric flask and dissolved and diluted up to the mark with acetonitrile to obtain a standard solution having concentration of LPV and RTV (1000 µg/mL). Accurately measured standard stock solution containing LPV and RTV (0.06, 0.2, 0.4, 0.8, 1.2, 2.0, 2.4 and 3.2 mL) were transferred in a series of 10 mL volumetric flasks and diluted with acetonitrile.

   The above solutions (50 µL) were spiked with 950 µL of drug free plasma to obtain LPV and RTV concentration of 300, 1000, 2000, 4000, 6000, 10000, 12000 and 16000 ng/ mL. The quality control samples were prepared in the same manner at three different concentration levels (low, medium and high) within the entire range of calibration.

2. **Extraction of sample:** Drug/metabolite free plasma samples with fixed aliquots of LPV and RTV (50 µL) and volunteer plasma sample (950 µL) was taken in glass centrifuge tubes and mixed with 1.0 mL acetonitrile for protein precipitation of plasma as well as 0.1 mL saturated sodium chloride solution. The samples were vortexes for 1.0 min., and then precipitated proteins were separated by centrifugation at 15000 rpm for 15 min. Supernant from the tube was collected and filtered through 0.45 µm nylon membrane filter before injection and then injected into the HPLC system.

3. **Method validation:** The proposed method was validated as per USFDA guideline with respect to linearity, repeatability, accuracy, precision, stability and specificity for its bio-analytical application.

RESULTS AND DISCUSSION:

For bulk and tablet dosage form:

1. **Linearity and range:** An aliquot (20 µL) of each calibration standard was injected under the operating chromatographic conditions. Chromatograms were recorded.

Methanol (20 µL) was injected under the same conditions and chromatogram of methanol was recorded for the correction of the response of methanol in the chromatograms containing responses of LPV and RTV. Calibration curves were constructed by plotting peak areas versus concentrations, and the regression equations were calculated. Each response was average of three determinations. Linear correlation was obtained between peak area and concentration of LPV and RTV in the range of 5-35 µg/ml., the linearity of the calibration curves were validated by the value of correlation coefficient of the regression (R^2) (**Table 1**).

2. **Accuracy (% Recovery):** Accuracy of the method was determined by calculating percentage recoveries of LPV and RTV by the standard addition method. Known amount of standard solutions of LPV (0, 5, 10 and 15 µg/mL) and RTV (0, 5, 10 and 15 µg/mL) were added to a pre-analyzed sample solution of LPV (10 µg/mL) and RTV (10 µg/mL). Each solution (20 µL) was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equations of the calibration curves. The percent recoveries were found in the range of 97.31-100.41 % and 98.29-101.54 % for LPV and RTV respectively, which indicated accuracy of the method (**Table 3**).

3. **Precision:** Repeatability was checked by repeatedly (n = 6) injecting the solution containing LPV (10 µg/mL) and RTV (10 µg/mL) and recording the chromatograms. Intra-day and inter-day precision was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 7 different concentration of LPV (5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 µg/mL) and RTV (5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 µg/mL). The results were reported in terms of % relative standard deviation. The % RSD for repeatability of LPV and RTV were found to be 1.84 and 1.62 respectively. The value of % RSD for intra-day precision was found to be in the range of 0.71-1.04% and 0.64-0.91% while inter-day precision was found to be in the range of 1.01-1.34 % and 0.95- 1.74% for LPV and RTV respectively, which indicated that the method was precise (**Table 4**).

4. **Limit of Detection and Limit of Quantification:** Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of intercept (N) and slope (S) of the calibration curve.

$$LOD = 3.3 \times N/S; \; LOQ = 10 \times N/S$$

The Limit of detection (LOD) was found to be 0.138 and 0.285 µg/ml while the Limit of quantification (LOQ) was 0.418 and 0.863 µg/ml for RTV and LPV respectively (Table 4).

5. **Robustness:** The robustness was studied by analyzing the samples of LPV and RTV by deliberate variation in the method parameters like changing the extraction time of LPV and RTV from tablet dosage form by ± 2 min, composition of mobile phase by ± 2% of organic solvents, wavelength by ± 2 nm, and flow rate by ± 0.2 mL/min.

   The changes in the response of LPV and RTV were noted and compared with the original one. The method was found to be robust as the results were not significantly affected by slight variation in extraction time, composition of mobile phase, and wavelength and flow rate of the mobile phase.

6. **System-Suitability Test:** System suitability tests were used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were retention time, tailing factor, resolution and theoretical plates of chromatographic peaks of LPV and RTV. The values of system suitability parameters for LPV and RTV are shown in **Table 5**.

7. **Specificity:** Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. The specificity of the method was determined by analyzing chromatogram of standard and sample solution. There was no interference from excipients or impurity found in determination of LPV and RTV indicated that the proposed method was specific (**figure 3 and 4**).

8. **Results of analysis of tablet formulation:** Analysis of tablet containing LPV 200mg and RTV 50mg was carried out and the amounts recovered were expressed as a percentage amount of the label claims. The percentage recovery of LPV and RTV was 98.99 ± 1.22 % and 99.67 ± 1.25 %, respectively (Table 2). The results are comparable with label claim.

For spiked human plasma study:

1. **Linearity and range:** An aliquot (20 µL) of each calibration standard was injected under the operating chromatographic conditions. Chromatograms were recorded. Drug free human plasma processed under the same extraction procedure as like spiked plasma (20 µL) was injected under the same conditions and chromatogram of drug free human plasma was recorded for the correction of the response of plasma in the chromatograms containing responses of LPV and RTV. Calibration curves were constructed by plotting peak areas versus concentrations, and the regression equations were calculated. Each response was average of three determinations. Linear correlation was obtained between peak area and concentration of LPV and RTV in the range of 300-16000 ng/ml., the linearity of the calibration curves were validated by the value of correlation coefficient of the regression (R^2) (Table 2).

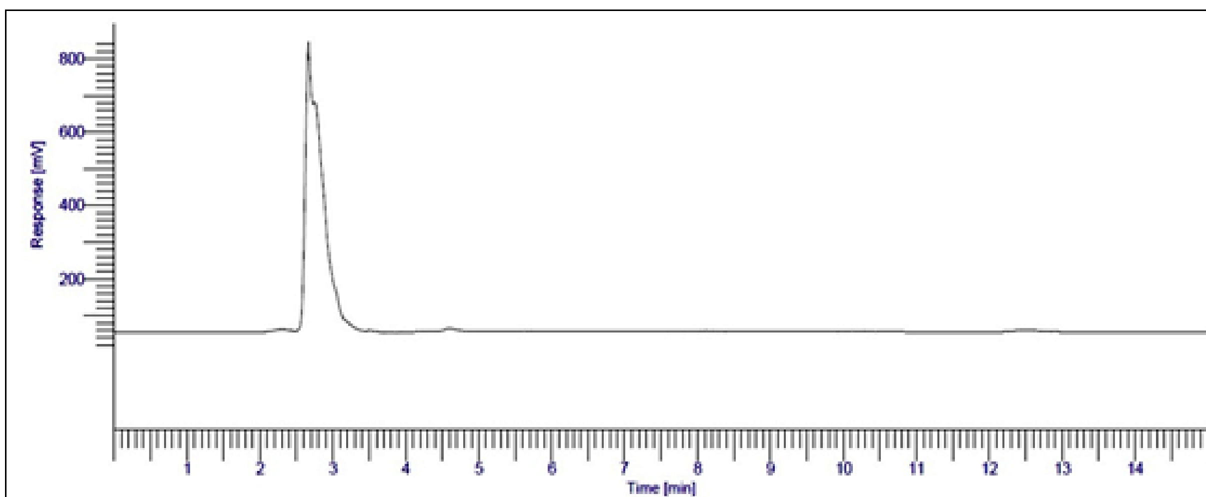
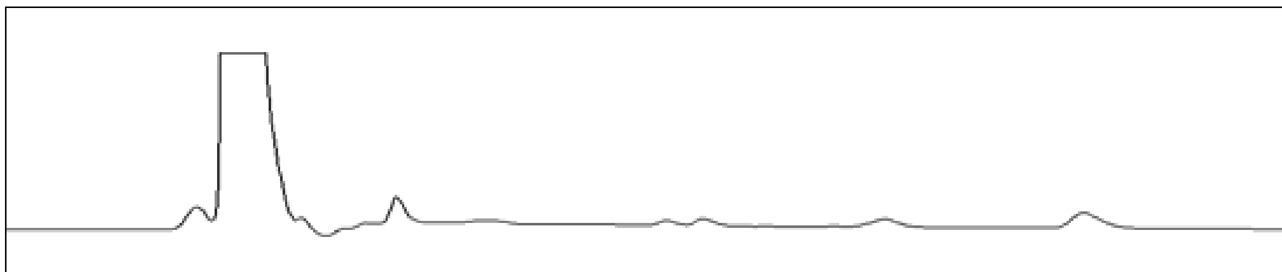


FIGURE 7: CHROMATOGRAM OF DRUG-FREE HUMAN PLASMA


ENLARGEMENT OF FIGURE 7

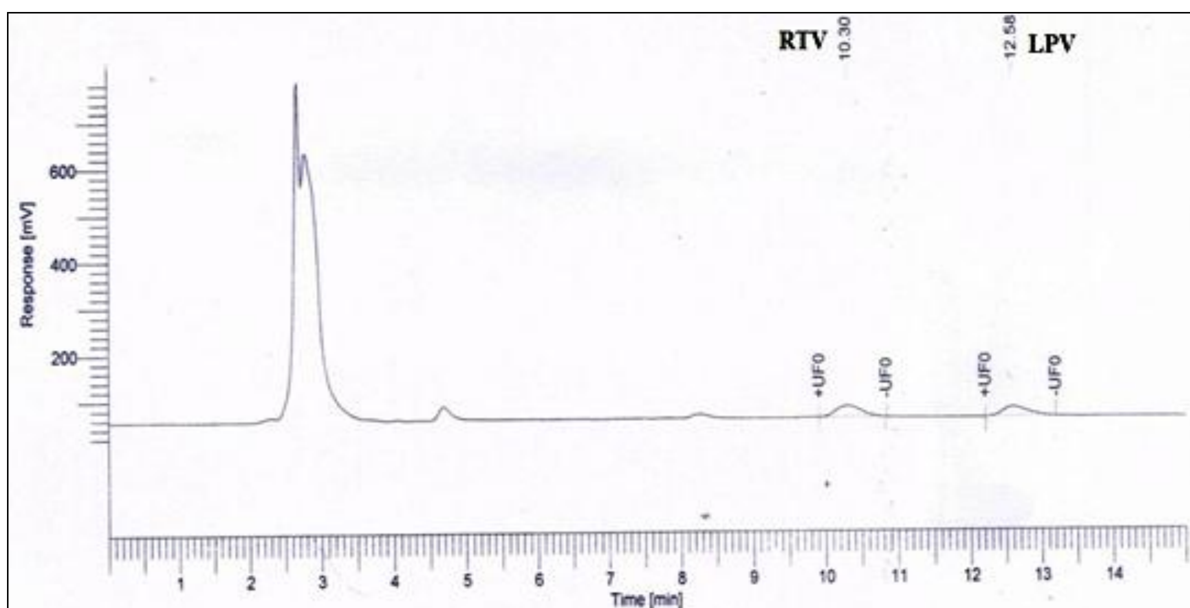
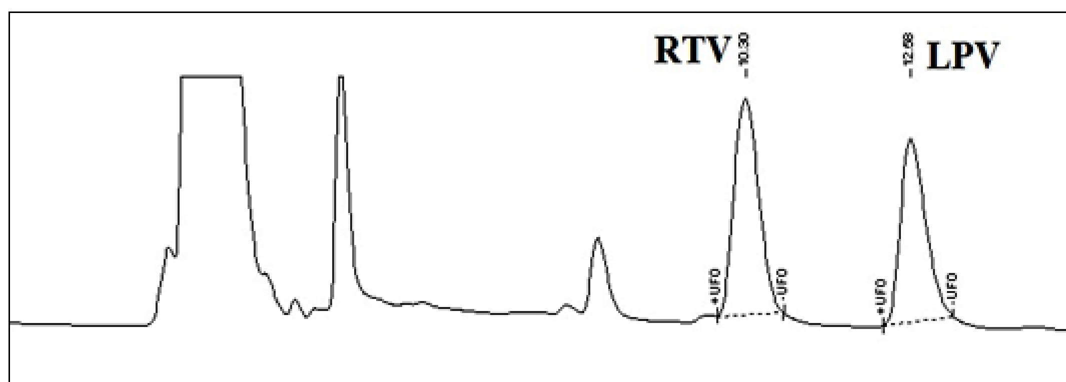


FIGURE 8: CHROMATOGRAM OF DRUG-FREE HUMAN PLASMA SPIKED WITH RTV AND LPV (16000 ng/mL) WITH CORRESPONDING RETENTION TIME AT 210 nm



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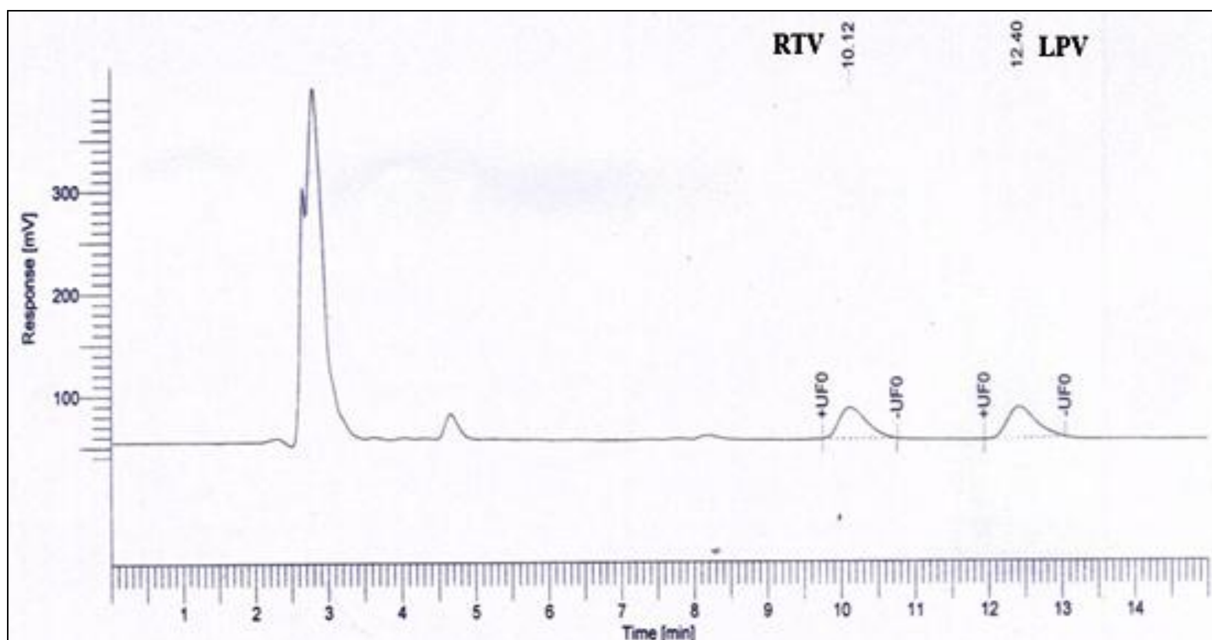
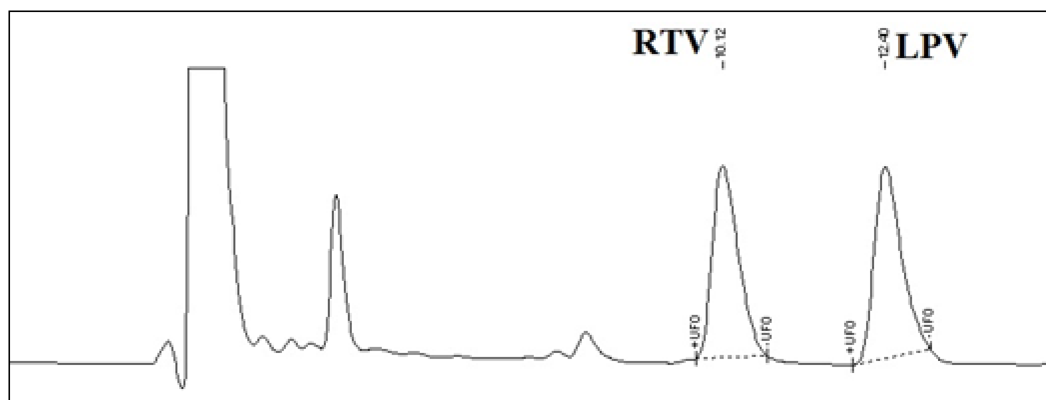


FIGURE 9 CHROMATOGRAM OF DRUG-FREE HUMAN PLASMA SPIKED WITH RTV AND LPV (12000 ng/mL) WITH CORRESPONDING RETENTION TIME AT 210 nm



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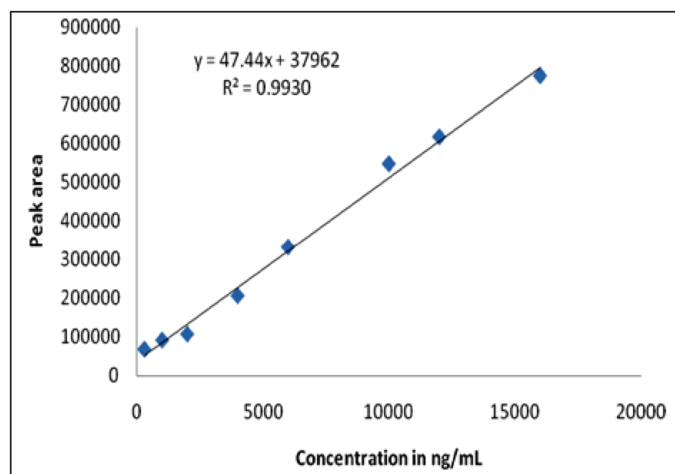

FIGURE 10: CALIBRATION CURVE OF RTV IN HUMAN PLASMA

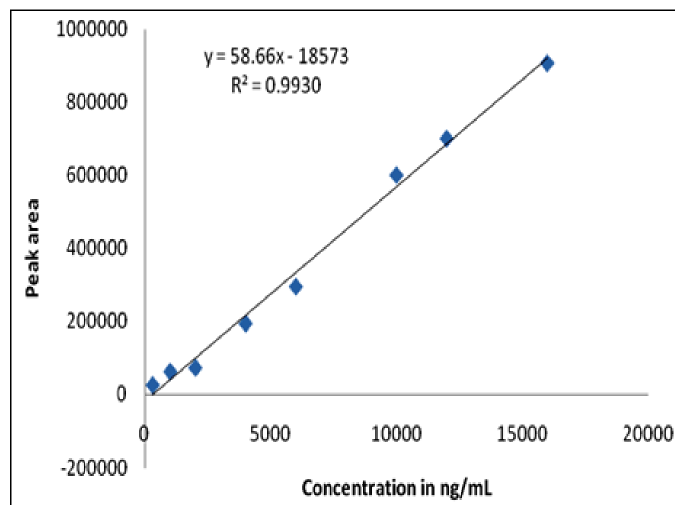

FIGURE 11: CALIBRATION CURVE OF LPV IN HUMAN PLASMA

2. **Accuracy:** Accuracy of the measurement of LPV and RTV in plasma was determined by standard addition method at four different concentration levels of LPV and RTV (6000, 8000, 10000 and 12000 ng/mL). The working solution (4000 ng/mL) was transferred to four different glass tubes. To each tube, 950 µL of drug/metabolite free plasma was added. Further 2000, 4000, 6000 and 8000 ng/mL of solutions were serially added into these tubes. These samples were extracted as described above in the extraction procedure. The percent recoveries obtained were 86.38 to 93.24% for RTV and 88.56 to 92.34% for LPV. The results of recovery study are given in **Table 7**.

3. **Recovery:** LPV and RTV recovery (relative and absolute) from human plasma were determined by spiking drug-free plasma (five replicates for each standard) with known amounts of the drug to achieve TNV concentrations of 300, 2000, 6000 and 12000 ng/mL. The spiked samples were processed and analyzed with the developed procedure. The relative (analytical) recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma with actual added amounts.

The absolute (extraction) recovery was obtained by comparing the observed peak areas obtained from the processed standard samples to direct injections of standard aqueous solutions prepared at concentrations which represented 100% recovery.

The absolute and relative mean recovery found to be 86.44% and 85.81% for RTV as well as 86.68% and 85.77% for LPV respectively (**Table 8**).

4. **Precision:** Method precision experiment was performed by preparing the working solution of LPV and RTV (4000 ng/ mL) for seven times and analyzed as per described under recovery. Relative standard deviation of peak area was found to be 9.44% for RTV and 10.66% for LPV (<15%) which indicated that the proposed method was repeatable. The intra and inter day precisions of LPV and RTV assay in human plasma were assessed from replicate samples spiked at four different concentrations (300, 2000, 6000 and 12000 ng/mL). The selection of concentrations for analysis was made according to definition of precision at low, medium and high concentrations of the linear range.

   The precision of the method is expressed as the relative standard deviation (% RSD) of the mean estimated concentrations. The intra-day precision (% RSD) ranged from 6.73 to 9.86% and 6.57 to 10.76% for RTV and LPV respectively while the inter-day precision (% RSD) ranged from 9.40 to 12.26% and 9.27 to 12.07% for RTV and LPV respectively (**Table 9**). The intra- and inter-day precision data showed that acceptable precision was obtained over the entire assay range.

5. **Limit of detection and limit of quantification:** The limit of detection (LOD) of RTV and LPV were found to be 67.86 ng/mL and 86.80 ng/mL, whereas, quantitative limit (LOQ) were 193.75 ng/ mL and 258.47 ng/ mL respectively.

6. **Stability:** The effect of frozen storage on LPV and RTV stability in human plasma was assessed through storing of LPV and RTV plasma samples at -70^{0}C over a period of 1 month. LPV and RTV plasma samples were analyzed immediately after preparation and at selected time intervals after storage over the study period. Stability was defined as <10% loss of initial drug concentration. Moreover, stability in heparinized plasma samples through five freeze and thaw cycles (-70°C ± 5°C to room temperature) has been confirmed.

   Samples, after thawing, were allowed to stand on the bench top, under room lighting till 2 hr had elapsed since their removal from the freezer. On the other hand , on-machine stability of the processed samples was evaluated by injecting the quality control samples, stored at room temperature in the HPLC, immediately after preparation and then at selected time intervals for 24 hr. The stability results showed that LPV and RTV were stable for at least 4 weeks when kept frozen at -70 ^{0}C. Thus, analysis up to 4 weeks storage confirmed adequate LPV and RTV stability at this temperature. Moreover, the results of freeze/thaw stability indicate that LPV and RTV were stable in plasma for at least five freeze/thaw cycles (**Table 10**).

7. **Specificity:** The specificity of the assay for the analyte in the presence of endogenous substances in the matrix was assessed by comparing the response obtained from the concentration used to produce the calibration plots with response obtained from six different human plasma samples. The percentage RSD was found to be less than 15% for the results of different human plasma samples.

TABLE 1: LINEARITY RESULTS IN BULK

Drug	Concentration range (µg/ml)	Equation for regression line	R^2
LPV	5-35	y = 34940x - 9443	0.9970
RTV	5-35	y = 26806x - 15165	0.9980

TABLE 2 ANALYSIS OF TABLET FORMULATION (n=3)

Drug	Labelled Amount (mg/tab)	Amount Found (mg/tab)	% Label Claim	S.D.	% R.S.D.
LPV	200	197.04	98.99	1.22	1.22
RTV	50	49.54	99.67	1.25	1.25

TABLE 3: DATA OF RECOVERY STUDY IN FORMULATION (n=3)

Drug	Amount taken (µg/ml)	Amount added (µg/ml)	Amount found (µg/ml)	Recovery ± SD, %	%RSD
LPV	10	0	9.82	98.24±1.21	1.21
	10	5	15.06	100.41±1.39	1.39
	10	15	19.94	99.71±0.97	0.97
	10	20	24.32	97.31±1.14	1.14
RTV	10	0	9.82	98.29±0.89	0.89
	10	5	14.75	98.34±1.46	1.46
	10	10	19.91	99.58±1.66	1.66
	10	15	25.38	101.54±0.79	0.79

TABLE 4: SUMMARY OF VALIDATION PARAMETERS IN BULK AND FORMULATION

Parameters	LPV	RTV
LOD (µg/ml)	0.285	0.138
LOQ (µg/ml)	0.863	0.418
Intraday precision (%RSD)	0.71-1.04	0.64-0.91
Interday precision (%RSD)	1.01-1.34	0.95- 1.74
Repeatability (%RSD)	1.84	1.62
Accuracy (%)	97.31-100.41	98.29-101.54

TABLE 5 SYSTEM SUITABILITY PARAMETERS IN BULK AND FORMULATION

Parameters	LPV	RTV
Tailing factor	1.18	1.20
No. of theoretical plates	6546.50	5334.77
Retention time	12.25 min.	9.90 min.
Resolution	4.095	-------

TABLE 6 OPTICAL AND REGRESSION CHARACTERISTICS IN HUMAN PLASMA

Parameter	RTV	LPV
Linearity range	300 - 16000 ng/mL	300 - 16000 ng/mL
Regression equation (y)	y = 47.44x + 37962	y = 58.66x - 18573
Correlation coefficient (R^2)	0.9930	0.9930

TABLE 7: DATA OF RECOVERY STUDY IN HUMAN PLASMA (n = 5)

Drug	Amount taken (ng/mL)	Amount added (ng/mL)	Amount recovered (ng/mL)	Accuracy ± SD%
RTV	4000	2000	5268.00	87.80 ± 6.30
	4000	4000	7172.00	89.65 ± 10.89
	4000	6000	9324.00	93.24 ± 7.71
	4000	8000	10365.60	86. 38 ± 10.53
LPV	4000	2000	5540.40	92.34 ± 8.28
	4000	4000	7084.80	88.56± 12.48
	4000	6000	9164.00	91.64± 7.23
	4000	8000	10491.60	87.43 ± 5.76

TABLE 8: ABSOLUTE AND RELATIVE RECOVERIES OF RTV AND LPV FROM HUMAN PLASMA

Drug	Nominal concentration (ng/mL)	Absolute recovery (mean ± SD%) (n = 5)	Relative recovery (mean ± SD%) (n = 5)
RTV	300	85.12 ± 8.83	84.28 ± 10.7
	2000	87.35 ± 5.43	86.82 ± 7.24
	6000	84.56 ± 9.57	88.42 ± 4.98
	12000	88.74 ± 11.64	83.74 ± 2.74
LPV	300	89.67 ± 11.4	85.32 ± 6.29
	2000	85.78 ± 9.54	83.37 ± 8.21
	6000	87.94 ± 7.58	89.13 ± 11.42
	12000	83.34 ± 5.51	85.28 ± 4.46

TABLE 9: INTRA AND INTER- DAY PRECISION OF RTV AND PLV IN HUMAN PLASMA

Nominal concentration (ng/mL)	RTV		LPV	
	Observed concentration. (ng/mL)	(% RSD)	Observed concentration. (ng/mL)	(% RSD)
Intra-day (n = 7)				
300	304.71± 30.04	9.86	294.22± 21.27	7.23
2000	1983.37 ± 133.48	6.73	2003.56 ± 215.58	10.76
6000	6022.45 ± 445.66	7.40	5888.71 ± 495.82	8.42
12000	11968.26± 1072.35	8.96	11976.34± 786.84	6.57
Inter-day (n = 7)				
300	289.23± 32.33	11.18	287.36± 29.79	10.37
2000	2004.34± 188.40	9.40	1989.58± 240.14	12.07
6000	5813.74 ± 572.07	9.84	6011.26 ± 626.97	10.43
12000	12014.38 ± 1472.96	12.26	11983.48 ± 1110.86	9.27

TABLE 10: SUMMARY OF STABILITY TESTING OF RTV AND PLV IN HUMAN PLASMA

Stability	RTV			LPV		
	Nominal conc. (ng/mL)	Observed conc. (ng/mL)	Precision (% RSD)	Nominal conc. (ng/mL)	Observed conc. (ng/mL)	Precision (% RSD)
Freeze/thaw (n = 5)	300	296.34	8.57	300	282.23	6.72
	2000	1981.66	6.90	2000	1957.38	9.45
	6000	5934.32	9.82	6000	5768.58	7.43
	12000	11575.49	3.45	12000	11354.39	10.36
Long term (n = 5)	300	276.12	7.53	300	271.34	8.30
	2000	1964.56	5.35	2000	1872.48	10.52
	6000	5781.42	10.63	6000	5739.45	6.37
	12000	11384.58	8.46	12000	11458.76	5.23

CONCLUSION: A simple, rapid, precise and accurate RP-HPLC method requiring no prior separation has been developed for the estimation of LPV and RTV in their combined tablet dosage form and applied to the drug spiked human plasma for bioanalytical development and validation. The developed method was found to be precise. Simple, accurate, repeatable and specific in bulk, dosage form analysis as well as spiked human plasma samples. The method can be employed for routine analysis of both the drugs alone, their combined tablet dosage form and for the determination of concentration of LPV and RTV in the human plasma for bioavailability and bioequivalence studies in clinical research.

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