Original Article

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Quantitative Determination of Lopinavir and Ritonavir in Syrup Preparation by Liquid Chromatography

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Abstract

A simple high-performance liquid chromatographic method (HPLC) was developed for the simultaneous determination of lopinavir and ritonavir in syrup. A reversed-phase with isocratic elution was utilized. The mobile phase was a mixture of 10 mM ammonium acetate, pH 7 and acetonitrile (50:50, v/v). The flow rate was operated at 1 ml/min and the presence of two interest compounds was detected by a UV detector at 245 nm. The retention times on the described method were about 12.5 min and 14.7 min for ritonavir and lopinavir, respectively. The developed HPLC method was also validated for important performance characteristics such as linearity, accuracy and precision. Linearity of the developed method was evaluated by using the correlation coefficient greater than 0.999 for both drugs. Accuracy was expressed as the percent of recovery and closed to 100%. Good precision was also obtained with the% RSD of less than 2 for all cases. The developed method was then applied to determine lopinavir and ritonavir in syrup sample. The percent labeled amount of both drugs obtained from HPLC method were 100.5% and 100.3% for lopinavir and ritonavir, respectively.

Key words: Lopinavir, Ritonavir, Protease inhibitors, HPLC

INTRODUCTION

Lopinavir and ritonavir (Figure 1) are protease inhibitors (PIs) which can prevent viral replication by inhibiting the activity of HIV-1 protease enzyme which is necessary to cleave nascent proteins for final assembly of new virus. These two drugs manufactured as a fixed combination by Abbott laboratories under the trade name of Kaletra® and this combination was approved by US FDA in September 2000. Lopinavir is approximately ten times more potent than ritonavir to fight against wild-type HIV. For ritonavir, it is a potent cytochrome P450 (CYP3A) inhibitor and usually used as pharmacokinetic booster for other PIs including lopinavir[1]. To analyze these drugs and other PIs, several determination methods have been reported. These included high-performance liquid chromatography-mass spectrometry (LC/MS)[2-5], matrix-assisted laser desorption/ionization, tandem time-of-flight (MALDI-TOF)[6], high-pressure thin layer chromatography (HPTLC)[7] and immunoassay methods[8]. This study aims to develop a simple liquid chromatographic method with UV detection as an alternative method for the determination of lopinavir and ritonavir in syrup.

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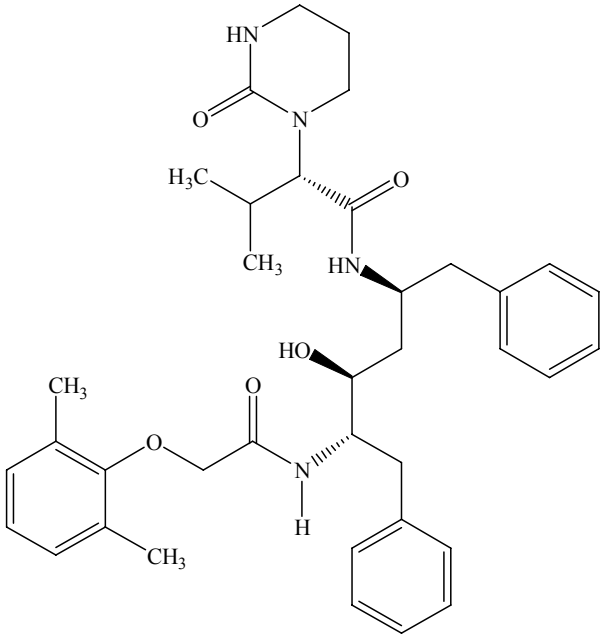
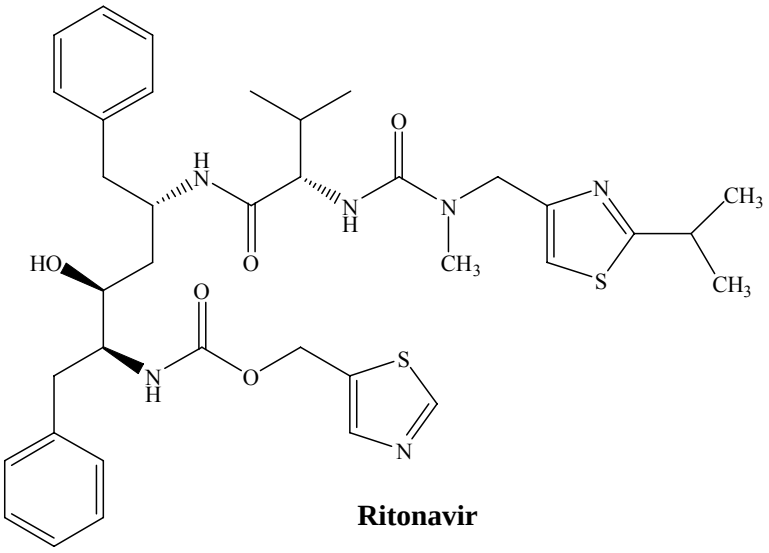


Lopinavir



Ritonavir

Figure 1. Chemical structures of lopinavir and ritonavir.

MATERIALS AND METHODS

Instrumentation

The chromatographic system was Shimadzu LC-10A (Shimadzu, Japan) consisted of LC-10AD pump, a SPD-10A UV-Vis detector, a class LC-10 work station and a Rheodyne 7125 injector with 20 µL loop (Rheodyne, USA). BDS Hypersil C18 (250 × 4.6 mm i.d., 5µm) supplied by Thermo Scientific (USA) was used for the separation of lopinavir and ritonavir.

Reagents and Chemicals

Standards Lopinavir, ritonavir, syrup sample and placebo syrup were kindly donated by The Government Pharmaceutical Organization (GPO, Bangkok, Thailand). Ammonium acetate (analytical grade) was purchased from RFCL limited (New Delhi, India). Acetonitrile (HPLC grade) was obtained from Lab-scan (Bangkok, Thailand) and deionized water was purchased from Thai Nakorn Patana Co.Ltd., (Bangkok, Thailand).

Standard preparations

Stock standard solutions of lopinavir and ritonavir at the concentration of 1 mg/ml were separately prepared by dissolving accurately weighed amount of the drugs in methanol. Working standard solution of ritonavir was prepared by dilution of its stock solution with 50% methanol until the final concentration of 0.1 mg/ml was obtained.

Preparation of calibration curves

The standard mixtures of lopinavir (2, 6, 10, 14 and 18 μg/ml) and ritonavir (0.4, 1.4, 2.4, 3.4 and 4.4 μg/ml) were prepared in 10 ml volumetric flasks by using lopinavir stock standard solution and ritonavir working standard solution. All standard mixtures were adjusted to volume with 50% methanol. Each standard mixture solution was injected into chromatographic instrument described above and the optimum mobile phase system. The calibration curve of each drug was separately plotted between concentrations (x-axis) versus corresponding peak areas (y-axis).

Sample preparation

Sample stock solution was prepared by dilution 100 μl of syrup sample with methanol in 10 ml volumetric flask. Then, 100 μl of this solution was transferred into 10 ml volumetric flask and made up to volume with 50% methanol. Desired concentration of sample was 8 μg/ml of lopinavir and 2 μg/ml of ritonavir in 50% methanol. This solution was filtered by using 13 mm, 0.45 μm nylon syringe filter before injecting into the HPLC column.

Method development

Standard mixture solution containing 32 μg/ml of lopinavir and 8 μg/ml of ritonavir was employed for HPLC method development. A HPLC method was developed utilizing a BDS Hypersil C18 (250 × 4.6 mm i.d., 5μm) HPLC columns. Mobile phase conditions such as type and concentration of organic solvents, effect of type, pH and concentration of buffer, were studied to obtain a suitable separation condition which yielded the lowest run time and acceptable chromatographic parameters such as resolution and peak shape. Two types of organic solvents, methanol and acetonitrile, were investigated for this approach. Various percent of acetonitrile were used to study the effect of organic solvent concentration. Effects of types and pH of buffer were studied with ammonium acetate and potassium phosphate. Mobile phase containing 10 mM of ammonium acetate or potassium phosphate buffer and acetonitrile (50:50, v/v) were prepared and studied. For effect of pH, a chosen buffer was investigated in the pH range of 3-8. The mobile phase flow rate was maintained at 1 ml/min through the study. The presence of interest compounds was detected at 245 nm with UV detector.

Validation of the method

Performance characteristics selected for method validation were linearity, accuracy, precision, specificity and range.

Linearity

Linearity was evaluated in the concentration range of 2-18 μg/ml for lopinavir and 0.4 - 4.4 μg/ml for ritonavir. The data were analyzed by least-squares linear regression method.

Accuracy

Accuracy of the developed method was studied by standard addition. The sample containing 4 μg/ml of lopinavir and 1 μg/ml of ritonavir was added to standard mixtures at the same concentration range for linearity study (2-18 μg/ml for lopinavir and 0.4 - 4.4 μg/ml for ritonavir). Accuracy was accessed in term of recovery percent of standards added.

Precision

Precision was investigated for both intra-day and inter-day precision. For intra-day precision, three concentration levels of standard mixtures (2, 10 and 18 μg/ml for lopinavir and 0.2, 2.4 and 4.8 μg/ml for ritonavir) were analyzed. Six determinations were done for each concentration on the same day. All solutions were injected in three replicates.

For inter-day precision, the same three different concentrations as for intra-day were studied on six different days and each concentration was triplicately injected. The precision of the method was expressed as the percentage of relative standard deviation (%RSD).

Specificity

Specificity was performed by using the chromatograms of unspiked sample, spiked drug substances (at 100% level) and placebo syrup. Sample containing ritonavir (2 µg/ml) and lopinavir (8 µg/ml) was used as unspiked sample. Standard mixture containing ritonavir (2 µg/ml) and lopinavir (8 µg/ml) was added to the same concentration of above sample and used as spiked sample. Chromatogram of placebo syrup was obtained by injecting the placebo at the same dilution as unspiked and spiked drug substances. All of the above solutions were prepared in 10 ml volumetric flask and 50% methanol was used as solvent.

Range

The upper and lower concentrations of the method were set up by the evaluation of linearity, accuracy and precision results.

RESULTS AND DISCUSSION

Method development

Effects of organic solvent

Effects of methanol and acetonitrile on the separation of desired compounds were investigated. Four mobile phases containing methanol or acetonitrile and water or 5 mM ammonium acetate buffer (50:50, v/v) were prepared and studied. No lopinavir and ritonavir peaks were observed in the mobile phase containing methanol and water (Figure 2A) or 5 mM ammonium acetate buffer (Figure 2B). The better chromatograms were obtained from the mobile phase containing acetonitrile, as shown in Figure 2 (C) and (D). Therefore, acetonitrile was selected for further studies.

The various percent (v/v) of acetonitrile (40, 45, 50, 55) in 5 mM ammonium acetate buffer, pH 7, were prepared and studied. As can be seen in Figure 3 (A), it took 60 minutes to elute lopinavir out off the column for the mobile phase containing 40% of acetonitrile. The best result for this study was obtained from the condition containing 55% acetonitrile. The retention times for this condition were about 8.8 minutes for ritonavir and 10.2 minutes for lopinavir. The good peak shapes were also achieved under this condition. However, to avoid the precipitation of buffer in high concentration of organic solvent, 50% acetonitrile was chosen for further studies.

Effects of types and pH of buffer

Two types of buffer, ammonium acetate and potassium phosphate, were studied. Mobile phase containing 10 mM of buffer and acetonitrile (50:50, v/v) were prepared and used to separate lopinavir and ritonavir. As illustrated in Figure 4, the shorten retention time of both drugs and good peak shapes were obtained from the mobile phase containing ammonium acetate buffer. Therefore, ammonium acetate was selected as a suitable buffer for further studies.

Selection of suitable buffer concentration is essential for chromatographic separation. High concentration of buffer has good efficiency to control pH but the precipitation in organic solvent can occur. Low concentration of buffer can avoid precipitation problem but this may result in the irreproducible retention time. Ammonium acetate buffer concentrations of 5-40 mM were evaluated for the effect on retention time of analytes. As seen in Figure 5, the mobile containing 20 mM ammonium acetate provided the shortest retention time for both drugs.

Effects of pH on the retention times of lopinavir and ritonavir are presented in Figure 6. Mobile phase containing 5 mM ammonium acetate and acetonitrile (48:52, v/v) was used to study the effect of pH. The pH of 5 mM ammonium acetate buffer was investigated in the pH range of 3-8. Both lopinavir and ritonavir posses low pK_a value, less than 3.7. As a result, it was found that an increase in the value of pH decreased retention time on both drugs. The pH 7 was selected as optimum pH for further studies.

Summary of chromatographic optimization

According to above studies, the optimal chromatographic condition was obtained. Lopinavir and ritonavir were separated on BDS Hypersil C18 column (250 x 4.6 mm i.d., 5µm). The mobile phase was 50:50 (v/v) acetonitrile and 5 mM ammonium acetate (pH 7). Isocratic elution was used and operated at the flow rate of 1 ml/min. The UV detection was 245 nm and the injection volume was 20 µl. Lopinavir and ritonavir were separated well on this condition with the retention time of 14.71 minutes and 12.47 minutes (Figure 7).

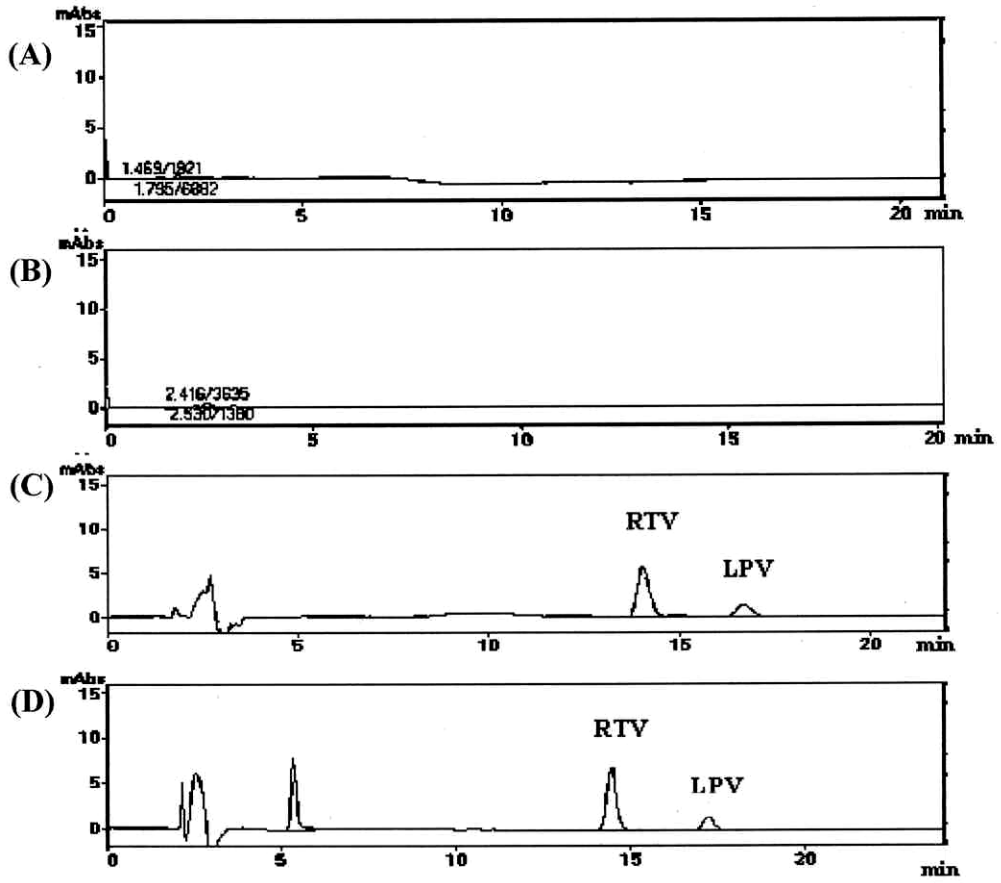


Figure 2. Chromatograms of lopinavir (32 µg/ml) and ritonavir (8 µg/ml) obtained from HPLC system with a BDS Hypersil C18 (250 x 4.6 mm i.d., 5µm) and mobile phase was (A) water and methanol (50:50, v/v); (B) 5 mM ammonium acetate buffer, pH 7 and methanol (50:50, v/v); (C) water and acetonitrile (50:50, v/v); (D) 5 mM ammonium acetate buffer, pH 7 and acetonitrile (50:50, v/v). The flow rate was 1 ml/min and UV detection was 245 nm.

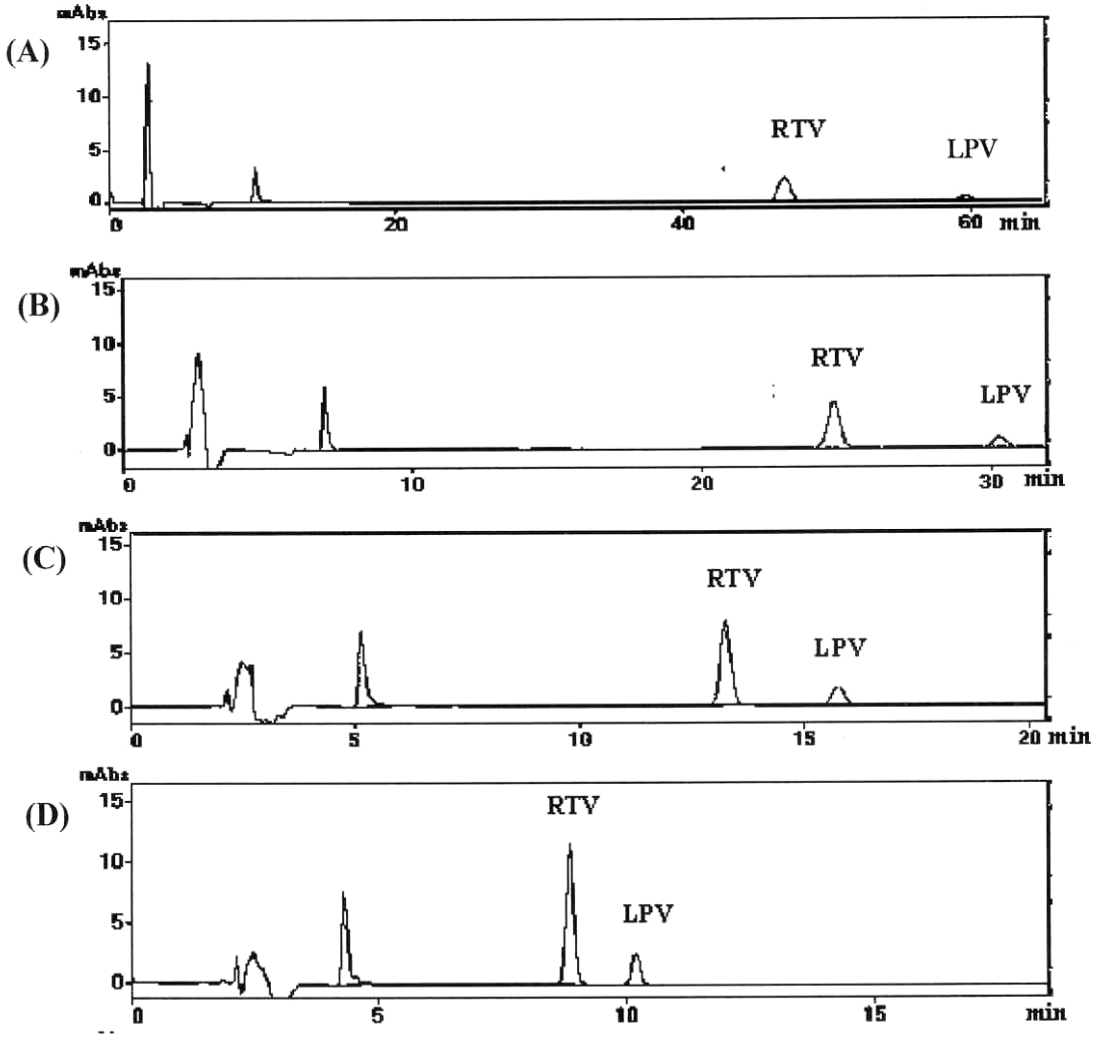


Figure 3. Chromatograms of lopinavir (32 µg/ml) and ritonavir (8 µg/ml) obtained from HPLC system with a BDS Hypersil C18 (250 x 4.6 mm i.d., 5µm) and mobile phase was (A) 5 mM ammonium acetate buffer, pH 7 and acetonitrile (60:40, v/v); (B) 5 mM ammonium acetate buffer, pH 7 and acetonitrile (55:45, v/v); (C) 5 mM ammonium acetate buffer, pH 7 and acetonitrile (50:50, v/v); (D) 5 mM ammonium acetate buffer, pH 7 and acetonitrile (45:55, v/v). The flow rate was 1 ml/min and UV detection was 245 nm.

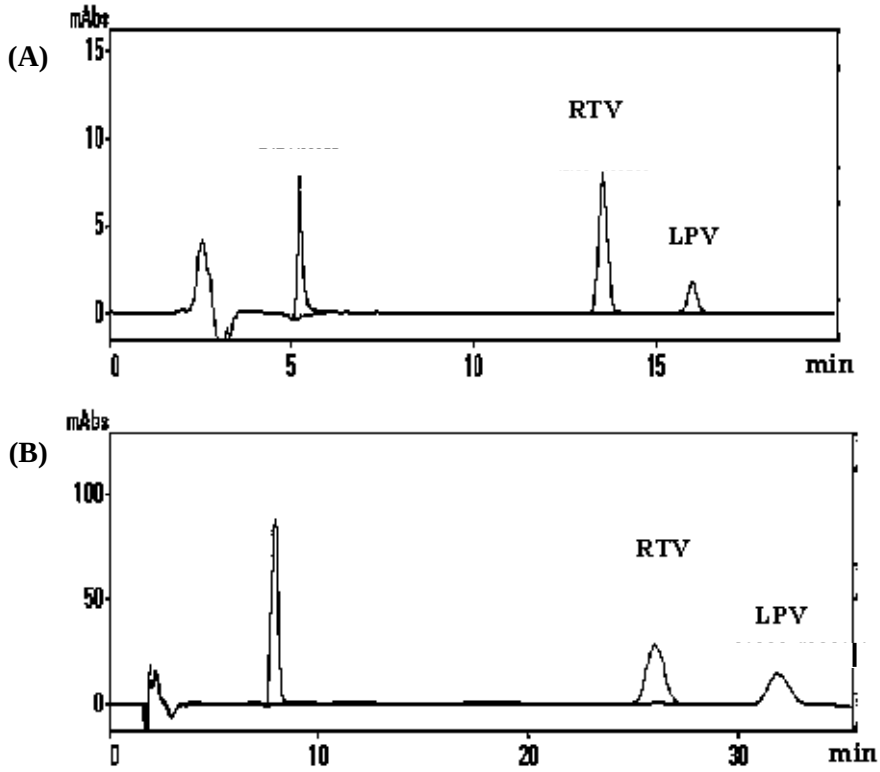


Figure 4. Chromatograms of lopinavir (32 µg/ml) and ritonavir (8 µg/ml) obtained from HPLC system with C18 column (250 x 4.6 mm i.d., 5µm) and mobile phase was (A) 10 mM ammonium acetate buffer, pH 7 and acetonitrile (50:50, v/v); (B) 10 mM potassium phosphate buffer, pH 7 and acetonitrile (50:50, v/v). The flow rate was 1 ml/min and UV detection was 245 nm.

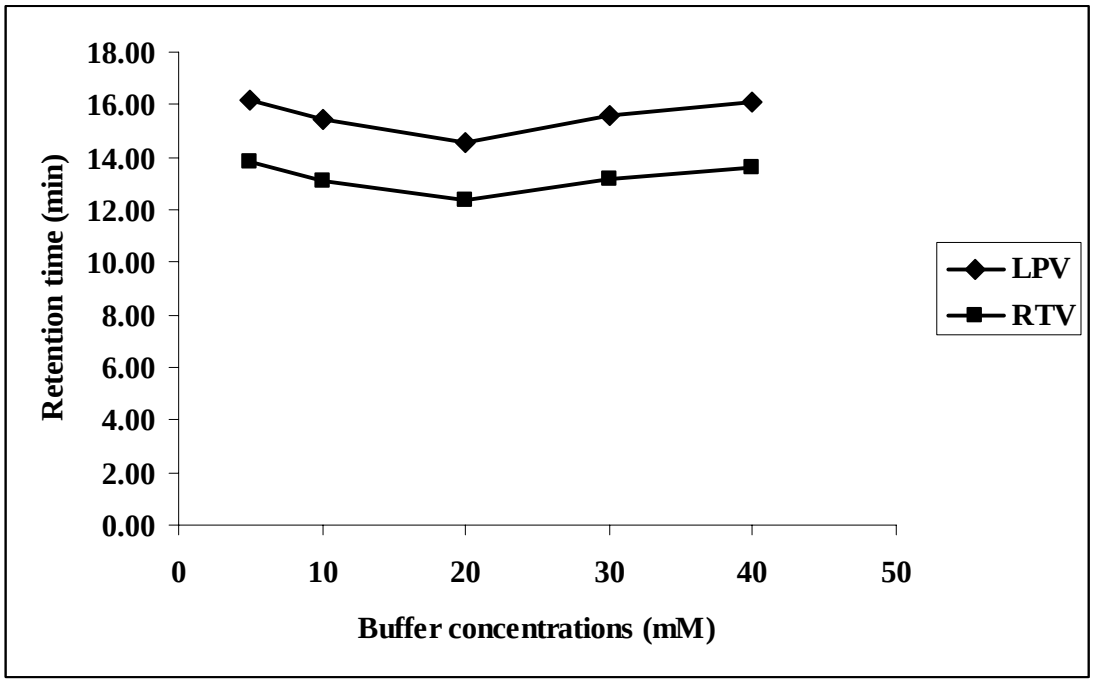


Figure 5. Effect of buffer concentration on the retention times of lopinavir and ritonavir.

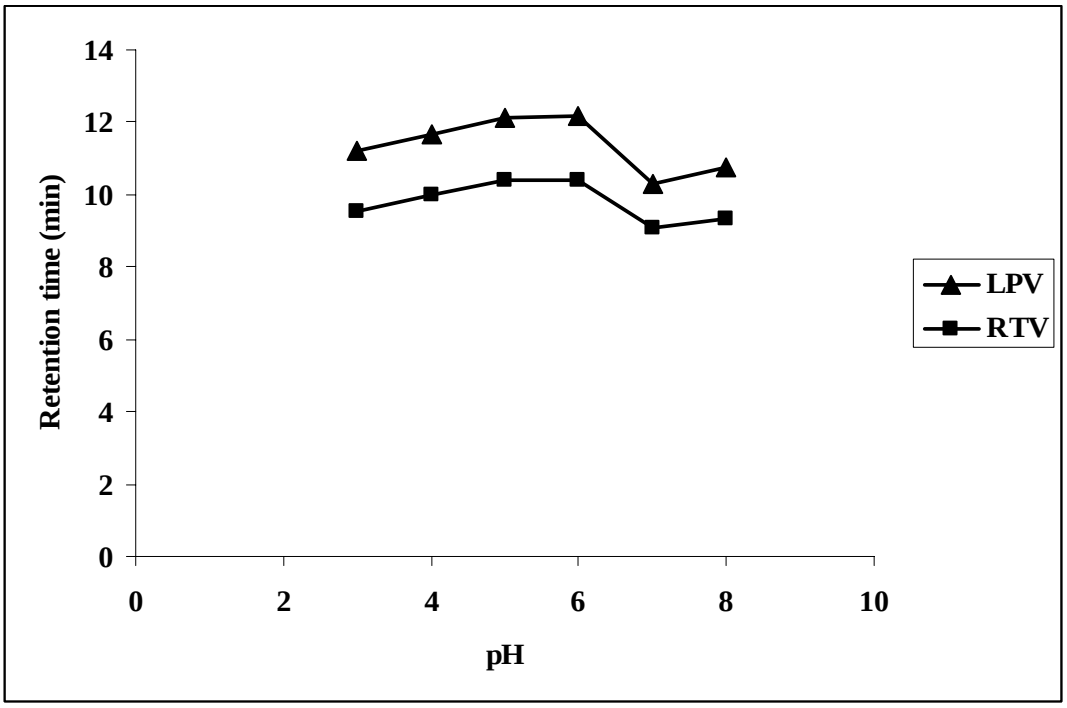


Figure 6. Effect of buffer pH on the retention times of lopinavir and ritonavir.

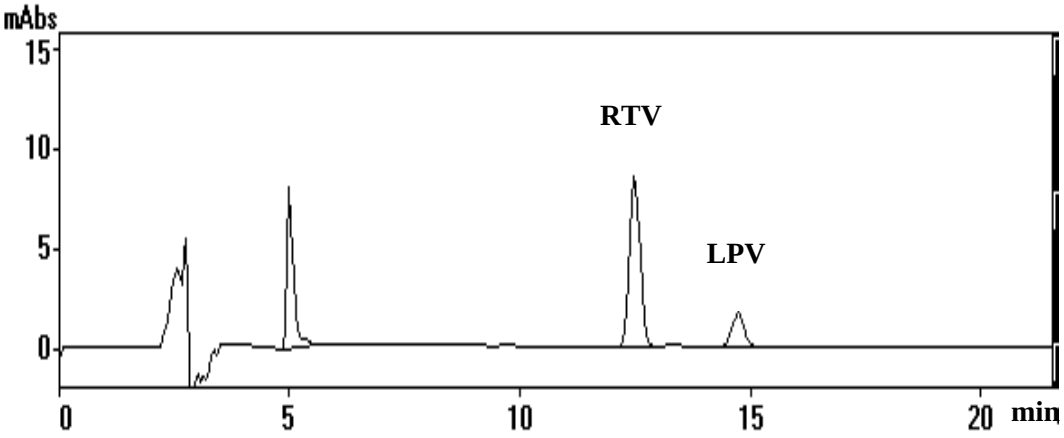


Figure 7. The chromatogram of lopinavir and ritonavir on the optimum chromatographic condition. HPLC column was BDS Hypersil C18 (250 x 4.6 mm i.d., 5µm) and mobile phase was 5mM ammonium acetate buffer, pH 7 with acetonitrile (50:50, v/v). The flow rate was 1 ml/min and UV detection was 245 nm.

Validation of the method

Linearity

The linear dependence of the peak area and concentration of lopinavir was evaluated in the concentration range of 2-8 μg/ml. Excellent linearity was obtained over the entire concentration range and correlation coefficient (r^2) was greater than 0.999. The relationship between concentration of lopinavir (x-axis) and peak area (y-axis) was y = 968.8x–203.6. For ritonavir, the calibration curve was linear over the concentration range of 0.4-4.4 μg/ml with the correlation coefficient (r^2) greater than 0.999. The relationship between concentration of lopinavir (x-axis) and peak area (y-axis) was y = 15653x + 669.07.

Accuracy

Accuracy was represented by the recovery percent of standard found and standard added to the sample. The mean value at each concentration level is shown in Table 1.The average recovery of lopinavir over the concentration range of 2-18 μg/ml was 98.9 to 99.9% and the average recovery of ritonavir over the concentration range of 0.4–4.4 μg/ml was 98.8 to 100.5%. Good recoveries of both drugs implied that the developed HPLC method was suitable for separation of desired drugs in syrup.

Table 1. Recovery data of standards lopinavir and ritonavir from spiked sample

Drugs	Concentration level (μg/ml)	% Recovery (mean ± SD, n = 3)
Lopinavir	2	99.65 ± 0.39
	6	99.69 ± 1.28
	10	99.94 ± 1.53
	14	98.91 ± 0.47
	18	99.42 ± 1.55
Ritonavir	0.4	100.07 ± 1.16
	1.4	98.77 ± 0.97
	2.4	99.41 ± 1.69
	3.4	99.87 ± 1.77
	4.4	100.51 ± 1.73

Precision

As summarized in Table 2, the intra-day precision of determination, as indicated by the relative standard deviation (RSD) value, for lopinavir ranged from 0.99 to 1.54% while the intra-day precision of ritonavir ranged from 1.20 to 1.97%. The inter-day precision for both drugs were less than 1.92 % over the entire concentration range 2–18 μg/ml for lopinavir and 0.4–4.4 μg/ml for ritonavir. These results showed that the proposed HPLC method yielded a satisfactory precision for analysis of lopinavir and ritonavir.

Specificity

Specificity was accessed in order to prove that the developed analytical method could select the interested analytes even though there were expected components such as impurities, degradation products and matrix components. Specificity was performed by demonstrating the chromatograms of unspiked sample, spike drug substances (at 100% level) and placebo syrup. All of these chromatograms are shown in Figure 8.

It was found that peak area of spiked drug substances (at 100% level) of both lopinavir and ritonavir were two times greater than unspiked sample. Chromatogram of placebo showed that there was not any peak presented at the same retention time of the desired compounds. It was indicated that syrup matrices did not interfere the determination of the interested analytes.

Table 2. Analytical data of intra-day and inter-day precision of standards lopinavir and ritonavir

Drugs	Precision	Concentration level (μg/ml)	% RSD (n = 6)
Lopinavir	Intra-day	2	1.54
		10	1.04
		18	0.99
	Inter-day	2	1.92
		10	1.53
		18	0.92
Ritonavir	Intra-day	0.4	1.20
		2.4	1.97
		4.4	1.40
	Inter-day	0.4	1.79
		2.4	1.75
		4.4	1.58

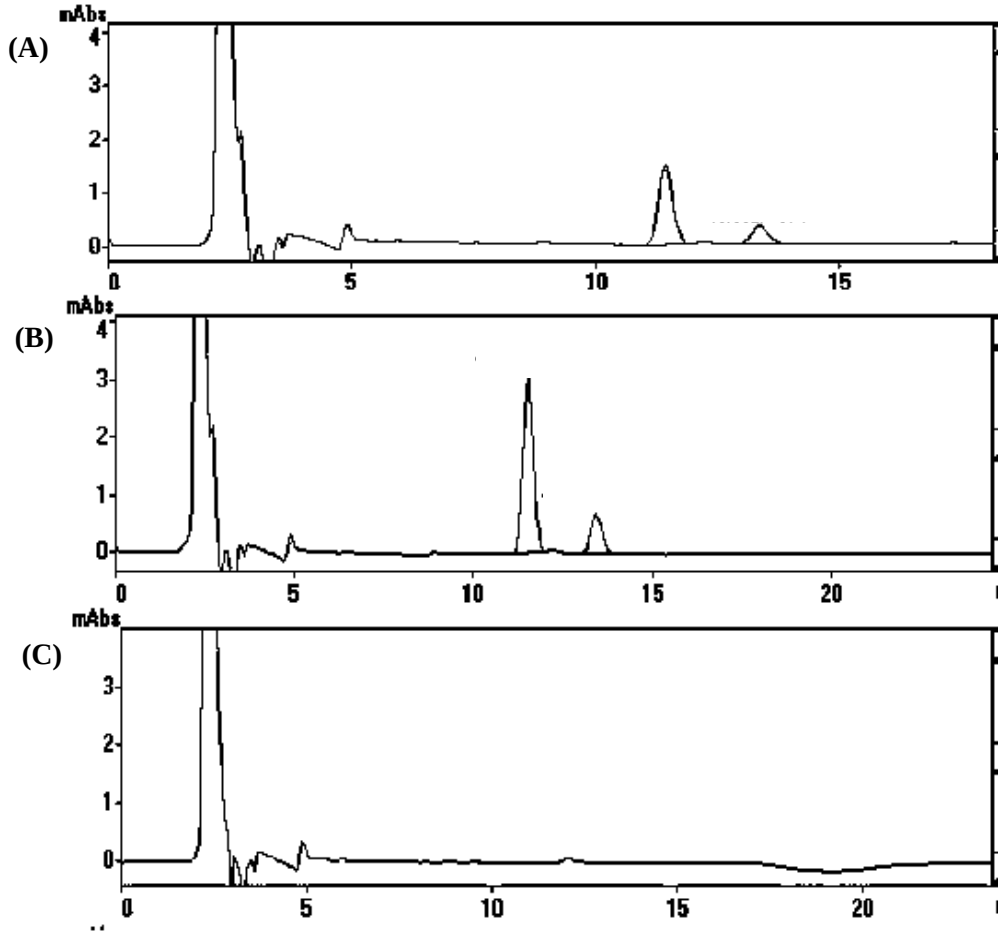


Figure 8. HPLC chromatograms of unspiked sample (A), spiked drug substances (B) and placebo syrup (C). Analytical column was a BDS Hypersil C18 (250 x 4.6 mm i.d., 5μm). Mobile phase was 20 mM ammonium acetate pH7 and acetonitrile (50:50, v/v). The flow rate was 1 ml/min, injection volume was 20 μL, detector was set at 245 nm.

Range

By evaluation of linearity, accuracy and precision results, it was found that the upper and lower concentrations with acceptable linearity, accuracy and precision were 2 and 18 μg/ml for lopinavir and 0.4 and 4.4 μg/ml for ritonavir.

Determination of syrup

The developed method was applied to determine lopinavir and ritonavir in syrup. The determination results were illustrated in Table 3. The results showed that the developed method can apply to real sample with good reproducibility.

Table 3. The concentrations of drugs in syrup sample obtained from HPLC method

Descriptions	Sample 1	Sample 2
Lopinavir		
Amount found (mg/ml)	80.10	80.64
Labeled claim (mg/ml)	80.00	80.00
% Labeled amount	100.1	100.8
Average	100.5	
Ritonavir		
Amount found (mg/ml)	20.00	20.10
Labeled claim (mg/ml)	20.00	20.00
% Labeled amount	100.0	100.5
Average	100.3	

CONCLUSION

In summary, a simple and rapid HPLC method was developed for the simultaneous determination of lopinavir and ritonavir in syrup. Dilution and filtration were only required for sample preparation. The excellent validation results were also achieved indicating the reliable of the developed HPLC method. In addition, the proposed method was applied very well to the real sample. This method may use as an alternative procedure for determination of other PIs drugs since its advantages were over the existing methods when the simple mobile phase and detector were utilized.

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