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Development and validation of dissolution test for lopinavir, a poorly water-soluble drug, in soft gel capsules, based on *in vivo* data

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ABSTRACT

The objective of the present study was to develop and validate a dissolution test for lopinavir soft gel capsules (Kaletra®), using a simulated absorption profile based on *in vivo* data. Different conditions such as surfactant concentration, apparatus and rotation speed were evaluated. *In vivo* release profiles were obtained from the literature. The fraction (and percentage) of dose absorbed (FA) was calculated by using Wagner–Nelson method. The best *in vitro* dissolution profile was obtained using Apparatus 2 (paddle) at 25 rpm, 1000 ml of medium with 2.3% of sodium lauryl sulfate and pH 6.0. Under these conditions a level-A *in vitro–in vivo* correlation (IVIVC) was obtained ($r = 0.997$). The *in vitro* dissolution samples were analyzed using a HPLC method and the validation was performed according to USP protocol. The method showed accuracy, precision, linearity and specificity within the acceptable range. Both the HPLC method and the *in vitro* dissolution method were validated and could be used to evaluate the release profile of lopinavir soft gel capsules.

1. Introduction

The level of interest in the *in vitro* dissolution of poorly water-soluble drugs has increased in recent years due to the need of finding a suitable dissolution media for pharmaceutical formulations that may reflect their *in vivo* performance [1]. IVIVC is defined as the correlation between *in vitro* dissolution and *in vivo* input rate and it may be used as an alternative for *in vivo* bioequivalence tests in order to minimize unnecessary tests with humans [2–4]. It may also be used instead of *in vivo* studies that may be required to demonstrate bioequivalence, when certain pre-approval and post-approval changes are performed in formulation, equipment, manufacturing process or in the manufacture site. However, in order to use *in vitro* dissolution test as an alternative for bioequivalence studies, the IVIVC must be predictive of the *in vivo* product performance [2–5]. The Biopharmaceutical Classification System (BCS) based on drug aqueous solubility and intestinal permeability proposed by Amidon et al. [6] may be used as a guide for setting *in vitro* dissolution specifications and also to foresee when an IVIVC may be successfully obtained. An IVIVC for immediate release solid oral dosage forms containing poorly water-soluble products may be established if the dissolution is the rate-limiting step in absorption [2,5,6].

In vivo poorly water-soluble drugs are solubilized through complex endogenous surfactants such as bile acids, bile salts and lecithin. However, *in vitro* dissolution models in less complex micelle systems have been used [1]. The use of surfactants in the dissolution system for poorly water-soluble drugs may be physiologically more meaningful due to the presence of natural surfactants in the gastrointestinal tract [7].

Lopinavir (LPV) and ritonavir (RTV) are two human immunodeficiency virus protease inhibitors. LPV is the antiviral component of Kaletra®, and the small amount of RTV present in the formulation does not contribute directly to antiviral activity, but it increases the oral bioavailability of LPV [8–10]. Considering that both drugs are practically insoluble, a dissolution test might be useful to predict the formulation *in vivo* performance [11]. Recently a dissolution method for RTV (Norvir®), based on its *in vivo* absorption profile was reported [12].

The objective of this study is to develop and validate a dissolution test for LPV (Kaletra®) soft gel capsules. The *in vivo* data was obtained from literature [13].

2. Materials and methods

2.1. Reagents

LPV and RTV standards (assigned purity of 99.5% and 98.7%, respectively) were kindly supplied by Cristália Produtos Farmacêuticos LTDA (São Paulo, Brazil). Kaletra® soft gel capsules

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(133.3 mg LPV and 33.3 mg RTV) were purchased from the market (batch no. 330962E21). Sodium lauryl sulfate (SLS) was obtained from Synth (São Paulo, Brazil). HPLC grade acetronitrile and methanol were obtained from Fischer Scientific (New Jersey, USA) and Merck (Darmstadt, Germany). Propylene glycol, oleic acid, polyoxyl 35 castor oil were obtained from Alfa Química (São Paulo, Brazil). All other reagents were analytical grade obtained from Nuclear (São Paulo, Brazil). Ultra-pure water (Milli-Q Plus, Millipore®, MA, USA) was used for the dissolution medium and throughout analysis.

2.2. In vivo study

The average plasma concentration versus time curve was fitted with a non-linear software (Micromath Scientist®, v.2.01) using a one-compartment open model, according to Eq. (1), and the resulting curve and parameters were used to estimate intermediate plasma concentration data points:

$$C = \frac{F \cdot D \cdot k_a}{V_d \cdot (k_a - k_e)} \cdot (e^{-k_e t} - e^{-k_a t}) \quad (1)$$

where C is the plasma concentration at time t, k_e and k_a are the elimination and absorption rate constants, respectively, V_d the distribution volume, D the dose and F is the bioavailability. The fraction of drug absorbed versus time plot was calculated using Wagner–Nelson method [14].

2.3. In vitro study

2.3.1. Dissolution test

The development and validation of the dissolution test was performed using a VANKEL® VK 8000 dissolution auto-sampling station, VK type bidirectional peristaltic pump, VK 750D digitally controlled heater/circulator, VK 7010 dissolution testing station multi-bath ($n = 8$) with automated sampling manifold. The influence of different SLS concentrations in the dissolution medium, different apparatus (USP basket and paddle) and different rotation speeds were evaluated. The medium volume used was 900 and 1000 ml and all tests were performed at 37 ± 0.5 °C. The dissolution medium pH was monitored before and after the performance of the tests. Sample aliquots were collected at 15, 20, 30, 45, 60, 90 and 120 min and assayed using HPLC.

2.3.2. Dissolution medium

The different dissolution media were prepared through simple SLS dissolution in ultra-pure water. The pH of all media was adjusted to 6.0 with 10% *o*-phosphoric acid. Acetate buffer (pH 4.4) was prepared by dissolving 10.9 g of sodium acetate trihydrate, 6.2 g of ammonium acetate and 20 ml of acetic acid in 1 l of water.

2.3.3. HPLC analysis

LPV and RTV were analyzed using a previously developed method [15]. Chromatographic analysis was carried out using a LC-20AT pump, CBM-20A system controller, SPD-10AV detector, SIL-20A auto sampler. Data integration was performed using Class-VP software for chromatographic peak integration (all from Shimadzu, Japan). For the specificity and stability studies, an SPD-M10ADVP photodiode array detector was used. The separation was achieved using a Merck C18 (5 μm, 250 mm × 4.6 mm i.d.) column. The mobile phase was composed of acetonitrile:water:methanol (53:37:10, v/v/v) at a flow rate of 1.2 ml min^{-1}. Detection was performed at 210 nm and all assays were performed at room temperature conditions. The auto sampler was programmed to inject 20 μl.

2.3.4. Solubility

LPV/RTV solubility was determined in three different media 0.1 M HCl, acetate buffer pH 4.4 and 2.3% SLS solution pH 6.0. Vessels containing 250 ml of medium were pre-heated at 37 ± 0.5 °C before adding the drugs in excess (133.3 mg of LPV and 33.3 mg of RTV). The samples were gently rotated in a heating chamber at 37 °C. Aliquots (5 ml) were removed from each vessel after 1 and 2 h, filtered, neutralized, diluted with the mobile phase and analyzed using the HPLC method. The solubility of Kaletra® was also evaluated through the same procedure. A capsule was placed in each vessel containing 250 ml of 2.3% SLS solution pH 6.0 The solubility in each medium was determined in triplicate.

2.3.5. In vitro–in vivo correlation

An IVIVC for LPV was evaluated by plotting the mean fraction of drug absorbed (FA) versus the mean fraction of drug dissolved (FD). Linear regression analysis was used to fit the data.

2.4. Validation of the dissolution test

The *in vitro* dissolution method developed was validated according to current guidelines [16–18]. Specificity, linearity, accuracy and precision were evaluated. LPV/RTV stabilities using test conditions were also evaluated.

2.4.1. Specificity

Specificity was evaluated in placebo samples. The placebo samples consisted of all the excipients (oleic acid, propylene glycol and plyoxyl 35 castor oil), sinkers and shell capsules without the active substance. The estimated concentration of excipients in Kaletra® was based on literature data [19]. The placebo sample were transferred to separate vessels ($n = 3$) filled with 1000 ml of dissolution medium at 37 ± 0.5 °C and stirred for 1 h at 150 rpm using paddle (USP Apparatus 2). Aliquots were collected and analyzed.

2.4.2. Linearity

Linearity of the method was studied through the injection of both LPV and RTV at the concentration range of 8–200 and 5–50 μg ml^{-1}, respectively, with five different concentration levels in each curve. Dilutions were performed with 2.3% SLS solution pH 6.0 from a methanolic solution containing 1600 μg ml^{-1} of LPV and 400 μg ml^{-1} of RTV. This study was conducted in 3 different days, and each solution was injected in triplicate into the HPLC system. The mean peaks area versus concentration data was treated by least-squares linear regression analysis. The relative standard deviation (R.S.D.) value for the slope and *Y*-intercept of the calibration curve was calculated.

2.4.3. Accuracy and precision

The accuracy of the method was evaluated through the recovery test of known amounts of LPV and RTV reference substance added to the placebo. A stock solution containing 10 mg ml^{-1} of LPV and 2.5 mg ml^{-1} of RTV was prepared in methanol. Aliquots of 4, 8 and 16 ml of this solution were added to vessels containing dissolution medium for a final volume of 1000 ml kept at 37 ± 0.5 °C (final concentrations were 40, 80 and 160 μg ml^{-1}, for LPV, and 10, 20 and 40 μg ml^{-1} for RTV, respectively). Samples were stirred at 150 rpm for 1 h. After that aliquots of each vessel were collected and analyzed. These studies were performed in triplicate on three different days. The same solutions used in the accuracy test were analyzed in order to ensure the precision of the method. Intra- and inter-day precision were established based on R.S.D. of the results.

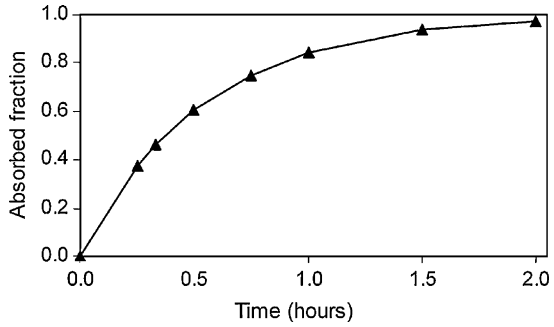

Fig. 1. Fraction of dose absorbed vs. time curve for lopinavir soft gel capsules (Kaletra®) using Wagner–Nelson method.

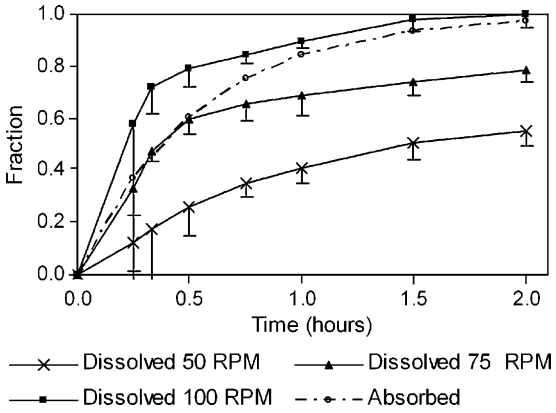

Fig. 2. Mean dissolution profiles of lopinavir from Kaletra® ($n = 8$) in 900 ml of 2.5% SLS as dissolution medium using basket in different rotation speeds compared with fraction absorbed using Wagner–Nelson method.

2.4.4. Stability studies

Stability of both drugs in the dissolution medium was evaluated using standards and samples. The solutions were kept at 37 ± 0.5 °C for 2 h under light shaking, being later left at room temperature for 24 h. Aliquots of the samples were tested at time 0, and after 2 and 24 h. The responses for the aged solutions were evaluated using a freshly prepared standard. The assay was performed in triplicate.

3. Result and discussion

3.1. In vivo study

The pharmacokinetic data for the IVIVC were obtained from the literature [13]. Using the Wagner–Nelson method, the average plasma concentration versus time curve was transformed into fraction of dose absorbed versus time (Fig. 1). Considering that the best fit for the *in vivo* data was obtained using an open one-compartment body model equation, the Wagner–Nelson method was used to obtain the fractions of dose absorbed.

3.2. In vitro study

3.2.1. Solubility of samples

Unlike RTV, LPV was rather insoluble in 0.1 M HCl. The highest solubility of ritonavir in low pH is due to protonation of the two weakly basic thiazole groups (pK_as 1.8 and 2.6). Lopinavir is a weak acid with pK_a of 2.8 and is not ionized in this pH [20,21]. The dissolution rate of both drugs in the dosage form was higher than the drug substances at the same conditions. Kaletra® soft gel capsule is an example of self-emulsifying drug delivery system (SEDDS), which is a technique used to improve the dissolution rate and hence the bioavailability [4].

Considering the volume of medium used in the tests and according to Table 1, the *sink* condition, defined as the volume of medium being at least three times higher than that necessary to obtain a saturated solution of the drug, was obtained with the SLS solution

Table 1
Solution solubility results

Medium	Sample	Solubility (μg ml^{-1})			
		Lopinavir		Ritonavir	
		1 h	2 h	1 h	2 h
0.1 M HCl	ds	5.4	13.7	132.1	133.3
Acetate buffer pH 4.4	ds	64.6	77.1	20.9	45.5
H_2O + 2.3% SLS pH 6.0	ds	458.7	477.2	112.8	118.9
H_2O + 2.3% SLS pH 6.0	Capsule	533.2	533.3	131.5	133.3

ds, drug substance.

2.3%, pH 6.0. It is believed that if the dissolution of the drug from its dosage form is the rate-limiting step for its absorption, then it will never saturate the medium in the gastrointestinal gut [1].

3.2.2. Dissolution profile of LPV—basket (USP Apparatus 1)

Using 900 ml of 2.5% SLS solution pH 6.0 as dissolution medium, three different rotation speeds were evaluated: 50, 75 and 100 rpm. Fig. 2 shows the mean dissolution profiles of LPV in Kaletra® capsules under these conditions ($n = 8$). At 50 rpm, the dissolution rate of LPV was too slow. Using 75 rpm, the *in vitro* dissolution profile was similar to the *in vivo* dissolution profile only at initial times. After 30 min, the dissolution rate was smaller than the absorption rate. At 100 rpm, unlike what was observed at 75 rpm, the dissolution rate of LPV up to 1 h was higher than the absorption rate. After this period, the dissolution rate was similar when compared to the absorption rate. Data at 15 or 20 min showed large R.S.D. (between 32 and 35%), which were attributed to differences in the opening times of the capsules. Afterward, R.S.D.s were smaller than 10%.

Under these conditions a poor correlation was established. Due to the higher or lower dissolution rate when compared to other *in vivo* data, other equipment was evaluated.

3.2.3. Dissolution profiles for LPV—paddle (USP Apparatus 2)

In these dissolution studies, using 50 rpm and 900 ml of medium containing three different SLS concentrations in water at pH 6.0 (0.5, 0.7 and 0.9%, w/v) were tested. Each experiment was performed with eight capsules. Although Kaletra® capsules did not float in the dissolution media, sinkers were used to keep them aligned with the paddle axis [16,18,22]. Fig. 3(a) shows the mean dissolution profiles of LPV (Kaletra®). Using 0.5% of SLS solution, LPV showed a very low dissolution rate. Increasing the concentration to 0.7% SLS, the dissolution rate was similar when compared to the absorption rate only up to 45 min. Using 0.9% SLS, LPV showed very high dissolution rate up to 45 min. However, in both SLS concentrations (0.7 and 0.9%, w/v), the dissolution rate was lower when compared to the absorption rate after 45 min.

It is possible to observe that the dissolution rate of LPV increases with the increase on the SLS concentration. According to Shah et al. [7,23] mediums containing surfactants solubilize poorly water-soluble drugs similarly to micelles from bile salts and lecithin, and increase the dissolution rate comparable to a hydro-alcoholic medium without using alcohol.

However, when using 50 rpm it was not possible to establish a good IVIVC. In order to obtain a better correlation, the following adjustments in the dissolution method were tested: dissolution medium volume was increased from 900 to 1000 ml; SLS concen-

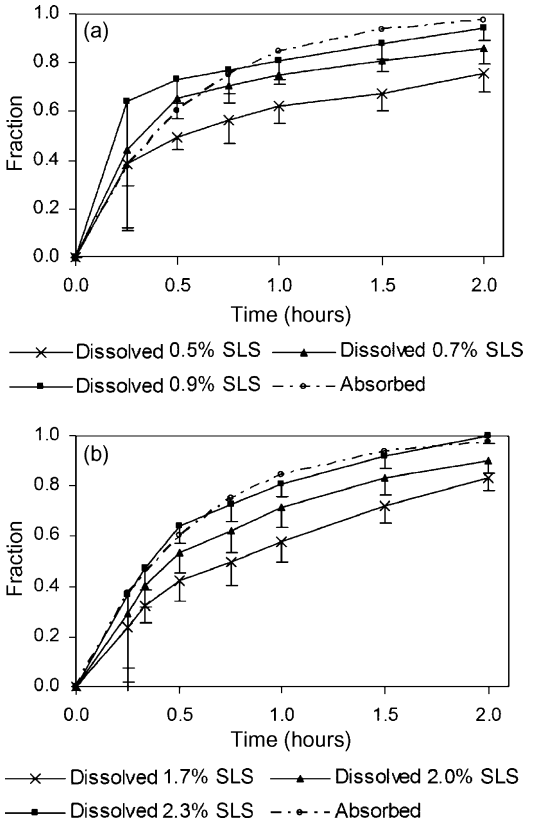

Fig. 3. Mean dissolution profiles of lopinavir from Kaletra® in three SLS concentrations using paddle at 50 rpm and 900 ml of dissolution medium (a) and paddle at 25 rpm and 1000 ml of dissolution medium (b), compared with fraction of drug absorbed (Wagner–Nelson method).

tration was changed and the rotation speed was reduced to 25 rpm. Three SLS concentrations were evaluated: 1.7, 2.0 and 2.3% and each assay was performed with 12 Kaletra® capsules. The rotation speed of 25 rpm is justifiable if it reflects better the *in vivo* performance [16–18].

The mean dissolution profiles of LPV in these conditions are shown in Fig. 3(b).

Data at 15 min from both experiments showed R.S.D. higher than 20% (between 21 and 31%) and thereafter R.S.D. were lower than 10%. It was possible to notice that the dissolution profile using 2.3% of SLS is similar to the *in vivo* absorption profile. The pH of the dissolution medium used is within physiological values as recommended for immediate release dosage forms [2,16,18]. There was no change in the pH of the dissolution medium before and after the test.

3.3. In vitro–in vivo correlation

The linear regression analysis of the data demonstrated that the use of 1000 ml of dissolution medium with 2.3% SLS at pH 6.0 and paddle at 25 rpm produced the best IVIVC and a level-A correlation was established ($r = 0.997$). The resulting equation (FA = −0.0019 + 1.0074FD) was used to back calculate FA in order to evaluate the accuracy of the model. The results are shown in Table 2. The level-A correlation is the highest correlation level possible, meaning that all the *in vivo* data points correlate well with the respective *in vitro* data points and thus one could be used to predict the other [2,3].

Table 2
Predicted fraction of lopinavir absorbed using the IVIVC equation (FA = −0.0019 + 1.0074FD)

Time (h)	FD	FA simulated	FA predicted	Error (%)
0.25	0.37	0.37	0.37	−1.4
0.33	0.47	0.46	0.47	2.4
0.50	0.64	0.60	0.64	6.1
0.75	0.72	0.75	0.73	−3.0
1.00	0.80	0.84	0.81	−4.0
1.50	0.92	0.94	0.92	−1.2
2.00	0.99	0.97	1.00	2.9

FA, fraction absorbed; FD, fraction dissolved.

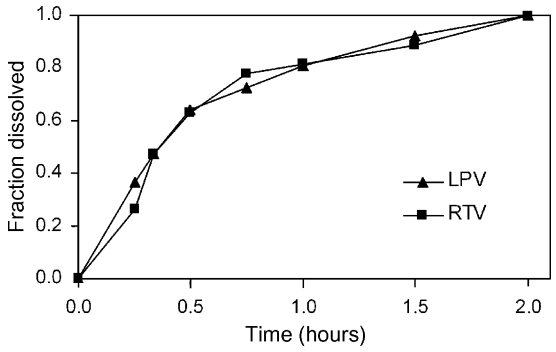

Fig. 4. Mean dissolution profiles of LPV and RTV from Kaletra® ($n = 12$) in 1000 ml of 2.3% SLS as dissolution medium using basket at 25 rpm.

3.4. Dissolution profile of RTV

The dissolution profile of RTV was similar when compared with LPV in the same conditions. The use of 2.3% SLS produced the best correlation for LPV (IVIVC) and in this condition the dissolution rate for RTV was similar to LPV (Fig. 4).

Although LPV is present in Kaletra® it does not contribute directly to antiviral activity but instead it inhibits CYP3A-mediated metabolism of RTV, improving its bioavailability [8–10].

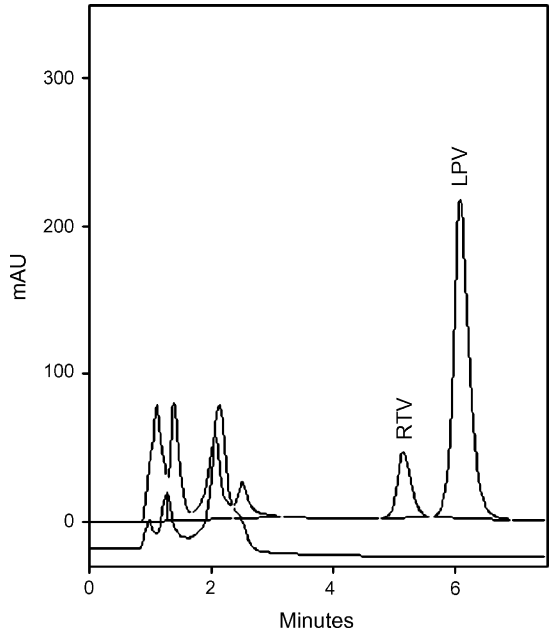

Fig. 5. The specificity of the method shows peaks of RTV and LPV from Kaletra® (top) and placebo sample (bottom) both in dissolution medium.

Table 3
Accuracy studies for lopinavir and ritonavir

Drug	Concentration added ($\mu g\,ml^{-1}$)	Concentration found ($\mu g\,ml^{-1}$) ± R.S.D. (%)	Recovery rate (%) ($n = 9$)
Lopinavir	40	40.0 ± 0.7	99.4–100.7
	80	81.3 ± 1.9	99.5–103.0
	160	162.5 ± 1.8	100.1–103.6
Ritonavir	10	10.3 ± 1.7	99.2–102.5
	20	20.6 ± 1.3	101.3–103.9
	40	40.8 ± 1.3	100.5–102.9

Table 4
Precision studies for lopinavir and ritonavir

Drug	Concentration added ($\mu g\,ml^{-1}$)	Intra-day ($\mu g\,ml^{-1}$) ± R.S.D. ($n = 6$)	Intermediate precision ($\mu g\,ml^{-1}$) ± R.S.D. ($n = 3$)
Lopinavir	40	40.3 ± 1.4	40.6 ± 0.6
	80	79.6 ± 1.2	80.8 ± 0.7
	160	161.3 ± 0.3	162.4 ± 0.7
Ritonavir	10	10.4 ± 1.7	10.0 ± 0.8
	20	20.3 ± 0.7	20.5 ± 0.8
	40	41.2 ± 1.3	41.5 ± 1.1

3.5. Validation of the in vitro dissolution profile

3.5.1. Specificity

No chromatographic peak from the placebo formulation was observed with the same retention time for both LPV and RTV (Fig. 5). LPV and RTV purities were higher than 0.999 and were obtained using a PDA detector, indicating that no interferences were observed. According to the USP Pharmacopeial Forum [16], the lack of chromatographic peaks from the placebo formulation demonstrates the specificity of the method.

3.5.2. Sample stability and standard solution

LPV and RTV were found to be stable under dissolution test conditions. The results demonstrated that sample and standard solutions remained at 100.0 ± 2.0% over a period of 24 h.

3.5.3. Linearity

LPV was linear in concentration range of 8 to 200 $\mu g\,ml^{-1}$. The mean (±R.S.D.) values of slope and *Y*-intercept were 35,444 (±0.68) and −26,252 (±8.94), respectively. The response for RTV was linear in concentration range of 5–50 $\mu g\,ml^{-1}$ with a slope of 28,256 (±0.58) and the *Y*-intercept at −7022 (±5.42), respectively. The correlation coefficients were >0.999 for both drugs. These results show there was a good correlation between the peak area and drugs concentration. The concentration range evaluated for both drugs included percentiles recommended for the dissolution test, from ±20% below the lowest expected concentration to ±20% above the highest expected concentration.

3.5.4. Accuracy and precision

The accuracy was demonstrated by the recovery of known amounts of LPV and RTV to the dissolution vessels. Recoveries from 95.0 to 105.0% of the added amounts are recommended in dissolution tests [16,18]. The mean recovery percentages for three different days ranged from 99.2 to 103.9% for both drugs (Table 3), corroborating the accuracy of the method. The intra-day precision was evaluated at three different concentration levels. The intermediate precision was evaluated in the same solutions at different days. Values presented in Table 4 show the good precision of the method with R.S.D. lower than 2%.

4. Conclusions

A level-A *in vitro–in vivo* correlation was established for lopinavir soft gelatin capsules (Kaletra®). The *in vitro* dissolution profile for lopinavir was obtained using 1000 ml of dissolution medium containing 2.3% of sodium lauryl sulfate in water at pH 6.0, USP Apparatus 2 at 25 rpm and 37 ± 0.5 °C. The equation that correlates fraction dissolved (FA) and fraction absorbed (FD) is: FA = −0.0019 + 1.0074FD. The validation results demonstrated that the *in vitro* dissolution method was accurate, precise, linear and specific. Both the HPLC analytical method and *in vitro* dissolution test were validated and could be used to evaluate the release profile of lopinavir soft gel capsules (Kaletra®).

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