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Determination of saquinavir and ritonavir in human plasma by reversed-phase high-performance liquid chromatography and the analytical error function

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Abstract

Two simple and reproducible high-performance liquid chromatography methods with ultraviolet detection were developed and validated for the quantitation of two protease inhibitors, saquinavir and ritonavir, in human plasma. The same single liquid–liquid extraction procedure with ethyl acetate–hexane (50:50, v/v), reversed-phase column and mobile phase were used. The analyses were accomplished using a Luna C_{18} column (150 mm × 4.6 mm i.d.) with a C_{18} guard column and, the mobile phase consisted of acetonitrile and 70 mM KH_2PO_4 adjusted to pH 5 with 80 mM Na_2HPO_4 (46:54, v/v). The wavelength was set at 240 nm for saquinavir and at 210 nm for ritonavir. The retention times were 6.4 min for saquinavir and 8.3 min for ritonavir. The methods were linear over the range of 100–2500 ng/ml for saquinavir and 200–2500 ng/ml for ritonavir. Intra and inter-day precision and accuracy were less than 10.2% for both drugs. Recovery were 90 and 87% for saquinavir and ritonavir, respectively. The drugs were stable at different relevant storage and working conditions. After the validation, their analytical error functions were established as standard deviation (S.D., ng/ml) = $4.84 + 7.14 \times 10^{-2}C$ (C is the theoretical concentration value) for saquinavir and S.D. (ng/ml) = $39.98 + 2.40 \times 10^{-5}C^2$ for ritonavir.

Keywords: Saquinavir; Ritonavir; Reversed-phase high-performance liquid chromatography; Human plasma; Analytical error function

1. Introduction

The treatment of the HIV infection improved at the end of 1995 with the appearance of the protease inhibitors (PIs) [1]. PIs, such as saquinavir and ritonavir, are widely used in combination with the reverse transcriptase inhibitors. This fact makes very hard regimens and, in consequence, the reduction of patient adherence to treatments. On the other hand, the low oral bioavailability of some PIs, the appearance of resistance, and the possible interactions with other concomitant drugs used to treat diseases associated to the HIV infection, may lead to insufficient plasma concentrations. For these reasons, drug monitoring of antiretroviral therapy may be an usual practice in most hospitals in order to avoid the failure of the treatments.

Several methods for individual determination of saquinavir and ritonavir have been described. They are mainly developed and validated for human plasma samples using reversed-phase liquid chromatography with ultraviolet detection after a pretreatment comprising protein precipitation [2], liquid–liquid extraction [3–5] or solid phase extraction [6]. However, most of these methods involved tedious and time-consuming sample preparation, which complicates routine analysis. Furthermore, none have reported the error function associated to the analytical method, although the measure of this error can be useful in some applications [7].

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Thus, two simple high-performance liquid chromatography (HPLC) methods with ultraviolet detection have been developed and validated to determine saquinavir and ritonavir in human plasma. After that, the error function associated to each analytical method, has been also established, which will be used as a possible weighting method in the pharmacokinetic parameter estimation by non-linear regression analysis.

2. Experimental

2.1. Chemicals and reagents

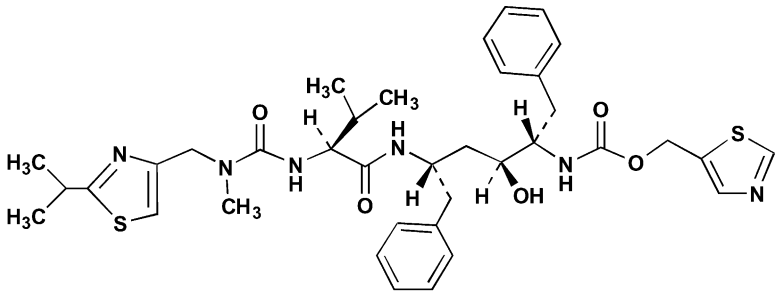
Saquinavir base and ritonavir were supplied by Roche Diagnostics GmbH (Mannheim, Germany) and Abbott Laboratories (Chicago, USA), respectively. Their chemical structures are represented in Fig. 1. HPLC-grade acetonitrile, methanol, and ethyl acetate were purchased from Carlo Erba (Barcelona, Spain). Hexane, sodium hydroxide, potassium di-hydrogen phosphate, potassium chloride, and di-sodium hydrogen phosphate anhydrous were obtained from Panreac Química (Montcada i Reixach, Barcelona, Spain), and boric acid was supplied from Probus (Badalona, Barcelona, Spain). All aqueous solutions including the HPLC mobile phase were prepared with purified deionized water (Milli-Q Plus, Millipore, Barcelona, Spain). Drug-free plasma was obtained from the Hospital General of Granollers blood bank (Granollers, Barcelona, Spain).

2.2. Chromatographic and detection conditions

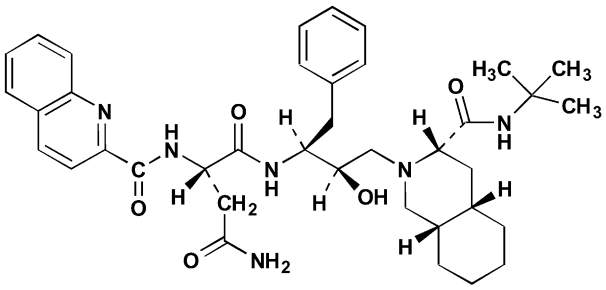
The chromatographic system consisted of a Waters (Waters Cromatografía, Cerdanyola del Vallès, Spain) equipped with a 717 plus autosampler, a 600 controller pump, an in-line degasser module and a 996 photodiode array detector. Data were acquired and processed using a Millennium software (ver. 2.15.01). The chromatographic separation was performed with a Luna C18 reversed-phase column (5 μm and 150 mm × 4.6 mm i.d.; Phenomenex, Barcelona, Spain), with a C18 guard column (ODS, octadecyl; 4 mm × 3.0 mm i.d.). The analysis of the samples was performed at room temperature (25 ± 2 °C). The mobile phase consisted of acetonitrile and 70 mM KH_2PO_4 adjusted to pH 5 with 80 mM Na_2HPO_4 (46:54, v/v) and was delivered at a flow rate of 1 ml/min. The solution was filtered through a 0.45 μm membrane filter and ultrasonically degassed prior to use. The photodiode array detector analysed a range of 200–300 nm, but the wavelength was set at 240 nm for saquinavir and 210 nm for ritonavir.

2.3. Preparation of stock solution, calibration standard and quality control samples

Stock solutions (50 μg/ml) of saquinavir and ritonavir were prepared by dissolving the appropriate amount of PIs, accurately weighted, in methanol–water (50:50, v/v). Aliquots of the stock solutions were stored at −20 °C until use. All stock solutions were found to be stable for, at least, 24 h at room temperature and for, at least, 1 month at



(a)



(b)

Fig. 1. Chemical structure of protease inhibitors assayed.

−20 °C. The calibration standards were daily prepared by dilution of the stock solutions with drug-free human plasma to obtain the desired concentrations, which covered the range of 100–2500 ng/ml for saquinavir and 200–2500 ng/ml for ritonavir. For the quality control (QC) samples, concentrations at three different levels (low, medium, and high) were prepared, which were 250, 500, 1000 ng/ml for saquinavir and 400, 1250, 2500 ng/ml for ritonavir.

2.4. Sample preparation

The liquid–liquid extraction procedure was the same for both drugs. In a 10 ml glass tube 1 ml of plasma sample spiked with the drug was mixed with 1 ml of 0.1 M borate buffer adjusted to pH 10 with 0.1 M NaOH and 5 ml of a mixture of ethyl acetate–hexane (50:50, v/v). The solution was shaken with a rotary mixer for 10 min. The organic phase was then transferred into another glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dry residue was reconstituted in 260 μl mobile phase and transferred to an autosampler vial. An aliquot of 200 μl was injected onto the HPLC system for analysis.

2.5. Method validation

A similar method validation protocol was followed for both drugs and included linearity, intra and inter-day precision and accuracy, recovery and stability assays [8–12].

About the linearity assay, five different standard concentrations covered the range of the calibration curves, 100, 250, 500, 1000 and 2500 ng/ml for saquinavir, and 200, 400, 800, 1250 and 2500 ng/ml for ritonavir. Each standard concentration was analysed in replicates of 3.

The precision and accuracy assays were carried out by analysis of QC samples in replicates of five on the same day (intra-day assay) and repeated on five days (inter-day assay). Concentrations were determined using a calibration curve for each day. The coefficient of variation (CV) for the precision, and the bias for the accuracy were obtained. The limit of quantitation was also determined and defined as the low concentration of the calibration curve with a CV less than 20% and a bias within ±20%.

The specificity of the methods was determined by comparing chromatograms of drug-free human plasma from six individuals with chromatograms of plasma spiked with saquinavir or ritonavir.

The absolute recovery (extraction efficiency) of both PIs was established by comparing the peak area responses from extracted calibration standards in replicates of three with those of non-extracted standards, which represented 100% recovery.

The stability of both drugs in human plasma following three freeze–thaw cycles was assessed using QC samples. An evaluation of the long-term stability was established from the results of QC samples stored at −20 °C during 1 and 3 months. Since HIV-infected patient samples are routinely heated at 57 °C to inactivate the virus prior to handling, the stability of QC samples after 1 h at 57 °C was also determined.

2.6. Determination of the analytical error

The procedure used to obtain the analytical error function of each validated method was previously reported [13,14]. A calibration curve with three replicates of each standard concentration was prepared and analysed. This procedure was repeated on 6 different days. The mean and standard deviation (S.D.) of each found concentration of the calibration curve were calculated every day. After that, the S.D. obtained (as dependent variable) and the theoretical standard concentrations (as independent variable) were regressed using polynomial analysis in order to determine the best function that would relate both variables. A maximum of a third-grade polynom was considered, whose general equation is S.D. = $A_0 + A_1C + A_2C^2 + A_3C^3$.

3. Results and discussion

3.1. Chromatographic conditions and extraction

The selection of chromatographic conditions included the mobile phase, flow rate, time of the chromatogram and the wavelength.

Some of the reported methods used phosphate buffer and acetonitrile at different pH to elute PIs [15–17]. After testing different percentages and pH, final conditions (acetonitrile and 70 mM KH_2PO_4 adjusted to pH 5 with 80 mM Na_2HPO_4, 46:54, v/v) yielded satisfactory results regarding reproducibility and the retention time for both drugs. Although at a flow rate of 1 ml/min only 10 min of chromatogram time were enough to quantify the desired peaks, a total run time of 20 min was found suitable to prevent disturbing peaks and the loss of baseline after the injection of some samples.

A maximum ultraviolet absorption around 240 nm was observed with saquinavir in the conditions of the analysis. In the case of ritonavir, the maximum absorbance was close to 200 nm, however, 210 nm was chosen to improve selectivity.

To extract saquinavir and ritonavir from human plasma, plasma samples were converted to basic medium before using the extraction solution [5,15–19] so that PIs were in their molecular form. Then, different organic solvents and mixtures were tested. A solution of ethyl acetate–hexane (50:50, v/v) resulted in the best extraction for both drugs.

Representative chromatograms of the PIs in human plasma are shown in Fig. 2(a)–(d). As it can be seen, no endogenous interferences were observed in blank human plasma samples at the retention times of interest, which were approximately 6.4 min for saquinavir and 8.3 min for ritonavir. Besides, no interference are expected according to literature revised about reversed-phase HPLC method with UV detection developed to quantify other PI plasma concentrations [20].

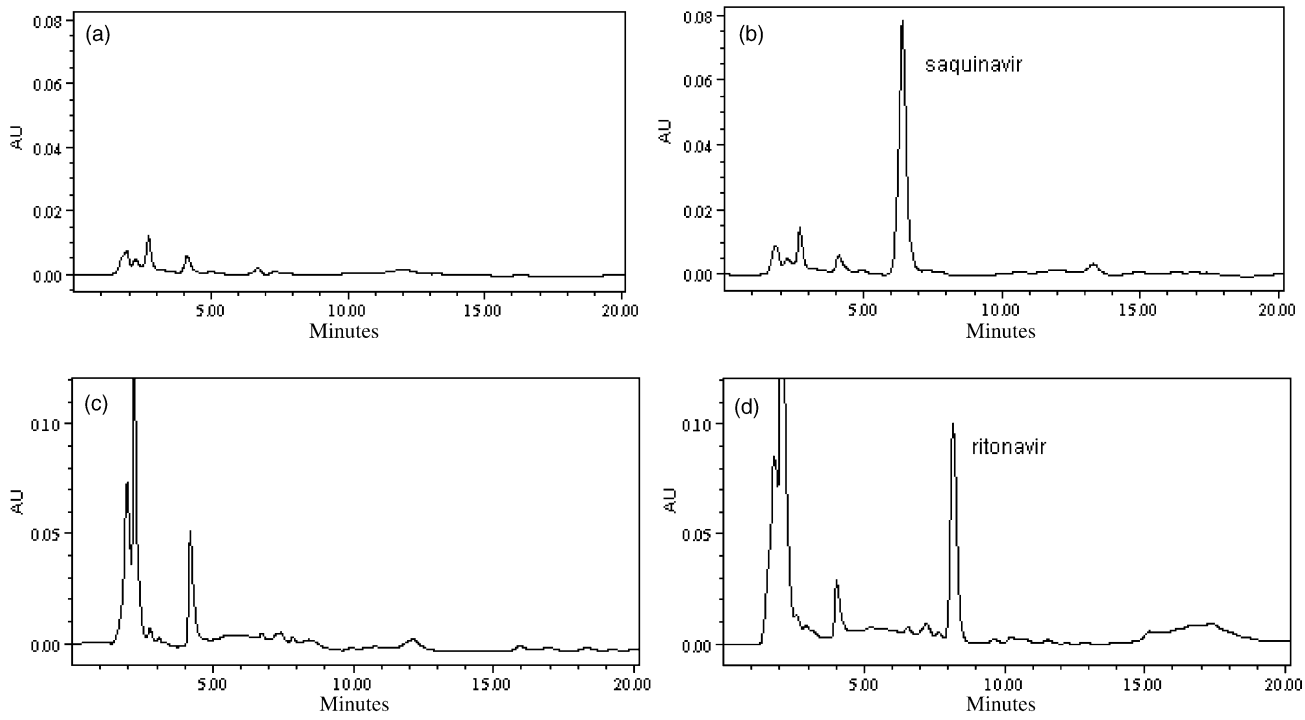


Fig. 2. Representative chromatograms of (a) drug-free plasma in condition of saquinavir method; (b) plasma containing 1000 ng/ml of saquinavir; (c) drug-free plasma in condition of ritonavir method; (d) plasma containing 1250 ng/ml of ritonavir.

3.2. Assay validation

In the linearity assay (Table 1), the CV of response factors was 6.2 and 10.7% for saquinavir and ritonavir, respectively. The mean calibration curve calculated by unweighed least-square linear regression analysis was: $y = 0.076 + 0.017x$, $r^2 > 0.9933$ for saquinavir and $y = -0.383 + 0.012x$, $r^2 > 0.9900$ for ritonavir, where y is the peak area and x the concentration. Thus, a good linear relationship between the peak area and concentration was observed over the entire range for both drugs.

Intra and inter-day precision and accuracy were within acceptable limits for both PIs (Table 2). Maximum CV values in intra-day precision were 9.5 and 10.1% for saquinavir and ritonavir and corresponded to the highest and lowest QC sample, respectively. Maximum CV values in inter-day precision were 4.1 and 5.7 for saquinavir and ritonavir, respectively, and corresponded to the lowest QC sample for both drugs.

The biggest bias were −7.0 and 4.9% in intra-day and −2.9 and −5.0 in inter-day accuracy for saquinavir and ritonavir, respectively, and corresponded to the lowest (intra-day) and medium (inter-day) QC sample (Table 2).

In the range of calibration standards, recovery in the plasma sample averaged 90 ± 5% for saquinavir and 87 ± 7% for ritonavir. The limit of quantitation was established in 100 and 200 ng/ml for saquinavir and ritonavir, respectively.

Saquinavir and ritonavir were shown to be stable for, at least, three freeze–thaw cycles, since there were no significant changes in concentration of QC samples during the experiment (Table 3). Evaluation of the stability of both PIs stored frozen at −20 °C during 1 and 3 months and at 57 °C during 1 h showed no significant change in concentration of the QC samples, as it is shown in Table 3. So, both drugs resulted to be stable in the laboratory conditions. The stability results obtained for both saquinavir and ritonavir are consistent with others reported previously [4,16].

3.3. Analytical error function

The best analytical error functions discriminated from the stepwise forward selection method were the following: S.D. (ng/ml) = $4.84 + 7.14 \times 10^{-2}C$ for saquinavir and $39.98 + 2.40 \times 10^{-5}C^2$ for ritonavir. Fig. 3(a) and (b) shows the fit of these functions to the mean values of S.D. obtained for

Table 1
Linearity assay for the quantitation of saquinavir and ritonavir in human plasma ($n = 3$)

Protease inhibitor	Linear regression curve	r^2	Standard error of the slope	F-ratio	P
Saquinavir	$y = 0.076 + 0.017C$	0.9933	3.3×10^{-4}	2653.9	0.0000
Ritonavir	$y = -0.383 + 0.012C$	0.9900	2.9×10^{-4}	1784.1	0.0000

Table 2
Intra-day (1 representative day) and inter-day precision and accuracy for the quantitation of saquinavir and ritonavir in human plasma

Protease inhibitor	Concentration added (ng/ml)	Intra-day assay (n = 5)			Inter-day assay (n = 25)		
		Concentration found (ng/ml)	CV (%)	Bias (%)	Concentration found (ng/ml)	CV (%)	Bias (%)
Saquinavir	250	267.4 ± 10.1	3.8	−7.0	252.8 ± 10.4	4.1	−1.1
	500	490.5 ± 33.9	6.9	1.9	514.4 ± 17.6	3.4	−2.9
	1000	983.0 ± 93.2	9.5	1.7	978.6 ± 8.1	0.8	2.1
Ritonavir	400	380.6 ± 38.5	10.1	4.9	388.2 ± 21.9	5.7	3.0
	1250	1296.3 ± 109.0	8.4	−3.7	1312.1 ± 44.2	3.4	−5.0
	2500	2469.3 ± 119.5	4.8	1.2	2446.9 ± 28.9	1.2	2.1

Concentration found are mean ± S.D.; CV (%) = (S.D./mean) × 100; bias (%) = [(concentration found − concentration added)/concentration added] × 100.

Table 3
Summary of stability assay for the determination of saquinavir and ritonavir in human plasma

Protease inhibitor	Concentration added (ng/ml)	−20 °C		Three freeze–thaw cycles (%)	57 °C, 1 h
		One month (%)	Three months (%)		
Saquinavir	250	97.8	97.7	96.8	98.1
	500	98.6	96.4	104.5	103.0
	1000	100.2	96.3	98.03	102.1
Ritonavir	400	96.7	95.4	97.5	96.0
	1250	102.2	97.2	101.2	98.0
	2500	98.2	96.1	102.9	96.9

% = percentage of concentration found.

each theoretical standard concentration (error bars represent the S.D. of the mean values on the 6 analysis days). Although both HPLC methods developed used similar reversed-phase column, mobile phase, detection system and extraction procedure, different models of error function were obtained, as it has been already reported in other studies with different drugs [21]. The error function associated to saquinavir analytical method was a first-grade polynom, showing a linear relationship between the S.D. and the theoretical concentration, whereas in the case of ritonavir was a second-grade polynom, showing a non-linear relationship. The error function (the reciprocal of the square of the standard deviation of any concentration value within the calibration range) will be used as a weighting method in non-linear parameter estimation in both pharmacokinetic studies and therapeutic drug monitoring. In this way, more precise measurements get greater weight, and less precise ones get less weight in the fitting process. Some notable examples of this approach have been reported with aminoglycoside antibiotics, vancomycin, digoxin, busulfan, lidocaine and other potentially toxic drugs, in which to be precise in order to optimise effectiveness is necessary [22–24]. Further studies are being planned for therapeutic drug monitoring of HIV-patients.

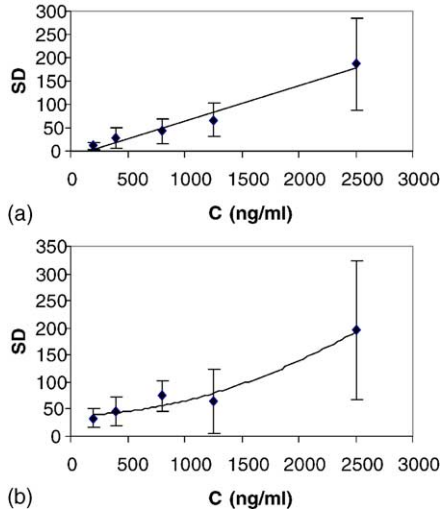


Fig. 3. Mean values and standard deviations obtained in the study of the analytical error function vs. theoretical standard concentrations from the calibration curves for (a) saquinavir and (b) ritonavir.

4. Conclusion

Two methods for the identification and quantitation of saquinavir and ritonavir in human plasma have been developed and satisfactorily validated over the concentration range selected. The methods described used similar liquid–liquid extraction procedure, mobile phase, reversed-phase column and detection system and can be used in hospital laboratories, where there is a great demand for simple and inexpensive chromatographic methods to determine saquinavir and ritonavir human plasma concentration.

The error function for each validated analytical method has been established by a convenient and practical procedure and will allow us to calculate the variance associated to a concentration value within the calibration range and to use it as a possible weighting method in non-linear regression analysis of pharmacokinetic studies in HIV-infected patients, especially in drug monitoring.

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