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Determination of saquinavir in human plasma, saliva, and cerebrospinal fluid by ion-pair high-performance liquid chromatography with ultraviolet detection

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Abstract

A high-performance liquid chromatographic method for the determination of the HIV protease inhibitor saquinavir in human plasma, saliva, and cerebrospinal fluid is described. Saquinavir was extracted from samples using C_2 extraction columns prior to ion-pair, reversed-phase high-performance liquid chromatography with ultraviolet detection at 239 nm. The method has been validated over the range of 2.5–4000 ng/ml using a 0.6-ml sample volume. This assay has been used for the analysis of saquinavir in plasma and saliva of HIV-1-infected patients. © 1997 Elsevier Science B.V.

Keywords: Saquinavir

1. Introduction

Saquinavir (Ro 31-8959, Invirase®, Fig. 1) belongs to a new class of antiretroviral drugs, the protease inhibitors, and is a potent in vitro and in vivo inhibitor of human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS) [1–4]. Saquinavir was the first member of its class to be approved by the FDA in the USA in 1995, under its accelerated approval regulations, for use in combination with approved nucleoside analogue reverse transcriptase inhibitors in patients with advanced HIV infection. The pharmacokinetic profile of saquinavir in HIV-infected patients has not yet been thoroughly investigated.

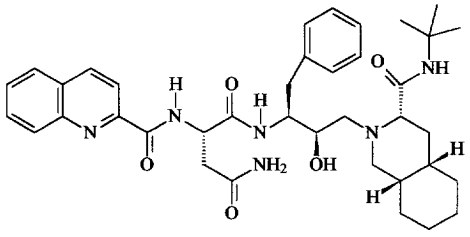

Fig. 1. Molecular structure of saquinavir.

A high-performance liquid chromatographic (HPLC) assay with mass spectrometric detection for the quantitation of saquinavir in human plasma has been described by Knebel et al. [5]. Their assay is

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linear in the range from 0.4 to 200 ng/ml. However, the bioanalytical method requires expensive equipment which is not readily available in most laboratories of hospitals caring for HIV-infected patients.

We report the development and validation of an ion-pair HPLC assay with ultraviolet detection for the determination of saquinavir in human plasma, saliva, and cerebrospinal fluid. This assay can be used to obtain pharmacokinetic data in HIV-infected patients.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Model 8800 solvent delivery pump (Spectra Physics, Santa Clara, CA, USA), a Model 8880 automatic sample injection device (Spectra Physics), a Spectra 200 programmable wavelength detector (Spectra Physics), and a Chromjet® integrator (Spectra Physics). The analytical column was a Zorbax® SB-C_{18} column (75× 4.6 mm I.D./particle size 3.5 μm; Rockland Technologies, Newport, DE, USA) protected by a Chromguard® C_{18} column (10×3 mm I.D.; Chrompack Nederland, Middelburg, Netherlands). Analytical runs were processed by the Autolab® Software Winner 386 System (Spectra Physics). UV spectra of saquinavir solutions in 50% (v/v) methanol were recorded with a Model 918 UV–Vis spectrophotometer (GBC Scientific Equipment, Dandenons, Australia).

2.2. Chemicals

Saquinavir mesylate (lot 40411001) was a kind gift of Roche Products, Research and Development (Welwyn Garden City, UK). Acetonitrile (HPLC supra-gradient) and methanol (supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Hydrochloric acid 37% p.a., ammonium acetate p.a., sodium acetate p.a., and hexane-1-sulfonic acid (sodium salt) p.a., were purchased from Merck (Darmstadt, Germany). Distilled water was used throughout. Blank, drug-free plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, The Netherlands). Blank saliva was provided by healthy volunteers. Blank cerebrospinal fluid was obtained from patients who underwent lumbar puncture for methotrexate monitoring after intrathecal administration of the drug.

2.3. Preparation of standards

Stock solutions of saquinavir were prepared by dissolving the appropriate amount of saquinavir mesylate, accurately weighed, in 50% (v/v) methanol to yield a final drug concentration (as base) of 1.0 mg/ml. For the construction of calibration curves fresh solutions were used. Drugs for interference analysis were obtained from the hospital pharmacy (Slotervaart Hospital, Amsterdam, The Netherlands), either as solutions for injections or after dissolving solid reference material in 50% (v/v) methanol (final concentration 500 μg/ml in 50% (v/v) methanol).

2.4. Sample pretreatment

For the preparation of the standard samples stock solutions of saquinavir were diluted with 50% (v/v) methanol. To achieve saquinavir calibration concentrations of 2.5–4000 ng/ml, appropriate quantities of the various diluted solutions were added to blank plasma, saliva, or cerebrospinal fluid in Eppendorf tubes (Merck). The solutions were mixed on a vortex mixer for 10 s. Next, 600 μl of plasma or cerebrospinal fluid were mixed on a vortex mixer for 10 s with 600 μl of a 0.1 *M* ammonium acetate solution. The tubes were then centrifuged for 3 min at 10 500 *g*, and 1.00 ml of the diluted samples was subjected to solid-phase extraction. To increase recovery from saliva samples, 600 μl of saliva was mixed with 600 μl of blank human plasma and 1200 μl of a 0.1 *M* ammonium acetate solution. Volumes of 2.00 ml of these mixtures were then, after centrifugation for 3 min at 10 500 *g*, subjected to solid-phase extraction.

Prior to solid-phase extraction, C_2 extraction columns (100 mg, 1 cc; Varian, Harbor City, CA, USA) were placed on a vacuum elution manifold (Baker 12-SPE System; J.T. Baker, Phillipsburg, NJ, USA), and rinsed with 1.0 ml of acetonitrile, followed by 1.0 ml of 0.1 *M* ammonium acetate

solution. The flow-rate was maintained at 1.0 ml/min. Care was taken that the columns did not run dry. Next, 1.0 ml of the diluted plasma or cerebrospinal fluid samples, or 2.0 ml of the diluted saliva samples were transferred onto the columns and drawn into them by applying reduced pressure. The columns were then washed with 1.0 ml of a mixture of acetonitrile and 0.1 *M* ammonium acetate solution (3:7, v/v), followed by vacuum suction for 1 min. Elution of the absorbed analyte was performed with 400 μl of a mixture of acetonitrile and 2.5 m*M* ammonium acetate solution (8:2, v/v) into Eppendorf tubes, and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residues were redissolved in 150 μl of mobile phase, mixed on a vortex mixer for 60 s and centrifuged for 3 min at 10 500 *g*. The clear supernatants were brought into autosampler vials with inserts.

2.5. Chromatography

The chromatographic analysis was performed at ambient temperature on a C_{18} analytical column with a mobile phase composed of acetonitrile plus water containing 25 m*M* sodium acetate and 25 m*M* hexane-1-sulfonic acid, and adjusted to pH 4.0 with hydrochloric acid 37% (40.5:59.5, v/v). Prior to use, air was removed by leading helium through the mobile phase. Absorbance was measured at 239 nm. The flow-rate was maintained at 1.0 ml/min. Aliquots of 100 μl were injected.

2.6. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank plasma, saliva, and cerebrospinal fluid samples. The following substrates were investigated for interference with the analytical method (including the sample pretreatment): didanosine, fluconazole, folinic acid, ganciclovir, indinavir, lamivudine, methadone, methotrexate, oxazepam, pyrazinamide, ranitidine, rifampin, ritonavir, stavudine, sulfamethoxazole, trimethoprim, zalcitabine, zidovudine, and zidovudine glucuronide in a final concentration of 20 μg/ml in plasma.

2.7. Limit of detection and limit of quantitation

The limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. At this concentration a significant difference between the spiked samples and the blank samples is required in plasma from six individuals (two-tailed, paired Student's *t*-test).

The lower limit of quantitation (LLQ) was investigated in plasma samples from six different donors. Each sample was spiked to contain 1, 2, 2.5 or 3 times the LOD concentration of saquinavir. For the concentration to be accepted as the LLQ, the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) are to be less than 20%. The upper limit of quantitation (ULQ) was arbitrarily defined as 4000 ng/ml.

2.8. Accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precision of the method were determined by assaying three replicate samples of plasma at three different saquinavir concentrations (88, 881, and 1537 ng/ml) in three analytical runs. Accuracy was measured as the percent deviation from the nominal concentration. The within-day and between-day precisions were obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable.

Linearity of three calibration curves was tested with the *F*-test for lack of fit, using a weight factor of (1/conc.) [6,7]. For the construction of each calibration curve 15 spiked plasma samples were analyzed in duplicate.

Recovery of saquinavir after the solid-phase extraction procedure was determined by comparing observed saquinavir concentrations in extracted plasma, saliva or cerebrospinal fluid to those of non-processed standard solutions.

2.9. Stability

Blank plasma samples were spiked with an aliquot of diluted saquinavir stock solution to give initial concentrations of 10.1, 101, and 1010 ng/ml. These samples were stored for 1 h at 60°C, 24 h at 25°C, 7

days at 4°C, and 30 days at −30°C. After the storage period four replicates of the samples were analyzed immediately.

2.10. Analysis of patient samples

Plasma and saliva from HIV-1-infected patients who ingested 600 mg saquinavir with a breakfast after an overnight fast were assayed for the protease inhibitor. Twelve heparinized blood samples were drawn during a time period of 8 h. Plasma was separated by centrifugation at 3000 *g* for 10 min at 4°C and was immediately stored at −30°C until analysis. Saliva samples were collected with a Salivette®-collecting device (Sarstedt, Etten-Leur, The Netherlands) by chewing on a cotton roll which contained 20 mg of citric acid to stimulate the saliva flow. Saliva was separated by centrifugation for 10 min at 3000 *g* at 4°C and was immediately stored at −30°C until analysis.

2.11. Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated *P* values were 0.05 or less.

3. Results and discussion

3.1. Chromatography and detection

Reversed-phase chromatography was initially performed with various mixtures of acetonitrile and 2.5 m*M* potassium dihydrogenphosphate solution at different pH values with a Zorbax® SB-C_{18} column (75×4.6 mm I.D./particle size 3.5 μm). To reduce the risk of tailing peaks, considering the basic character of saquinavir, we decided to use this end-capped analytical column.

Saquinavir demonstrated only minor fluorescence (wavelengths of excitation and emission are 325 and 375 nm, respectively), no explorable electrochemical properties, but significant ultraviolet (UV) absorption. Thus, for assaying saquinavir UV detection was performed at 239 nm since this was the wavelength of maximal absorption of saquinavir in the mobile phase (specific extinction, 835).

The chromatographic characteristics of saquinavir were highly dependent on the pH value of the mobile phase. After testing various phosphate and acetate solutions at different pH values, a 25 m*M* sodium acetate solution showed satisfactory results regarding the reproducibility of the retention time of saquinavir. The pH value of the sodium acetate solution was adjusted to 4.0 with a 4 *M* hydrochloric acid solution in distilled water. Peak shape and separation from endogenous compounds were further optimised by the addition of hexane-1-sulfonic acid. The concentration of hexane-1-sulfonic acid was optimised in the range from 6 to 50 m*M*. A concentration of 25 m*M* proved to be optimal regarding peak shape and separation from endogenous compounds.

We had no suitable internal standard available. The use of other HIV protease inhibitors with a similar molecular structure (such as indinavir or ritonavir) as an internal standard was not considered since these compounds are increasingly coadministered with saquinavir in HIV-infected patients. Furthermore, the assay as described gives satisfactory validation results without the use of an internal standard.

3.2. Sample pretreatment and recovery

Knebel et al. reported solid-phase extraction of 0.25 ml of human plasma with high recovery (>95%) with the use of C_2 extraction columns [5]. In order to increase the LLQ of our method we used 0.6 ml of the biological matrix.

Recovery of saquinavir from the solid-phase extraction columns was determined and calculated by comparing observed saquinavir concentrations in extracted samples to those of non-processed standard solutions. When saliva was used, an aliquot of 0.6 ml of blank plasma was added to 0.6 ml of saliva to increase the recovery of saquinavir. Without the addition of blank plasma the recovery from saliva was only 40%. Saquinavir did not adsorb to the cotton role of the Salivette® device that was used to collect saliva.

For plasma samples the recovery ranged from 90 to 94%, for saliva samples from 85 to 94%, and for cerebrospinal fluid from 60 to 61% (Table 1).

Table 1
Recoveries of saquinavir from spiked samples

Saquinavir concentration (ng/ml)	Recovery (mean±S.D.) (%)
Plasma[a]	
30	90±14
300	90±5
500	94±1
Saliva[b]	
30	85
300	87
500	94
Cerebrospinal fluid[b]	
30	60
300	61

S.D., standard deviation.
[a] Determined in four analytical runs.
[b] Determined in one analytical run.

3.3. Specificity and selectivity

Blank plasma, saliva, and cerebrospinal fluid from six different individuals showed no interfering endogenous substances in the analysis of saquinavir (Fig. 2A–C). Potentially co-administered drugs or metabolites tested had retention times that were different from saquinavir (indinavir, ritonavir, rifampin) or were not detected with the described bioanalytical method. The elution of endogenous compounds with a retention time of approximately 30 min necessitated a run time of 35 min.

3.4. Limit of detection and limit of quantitation

The LOD in plasma was 1.0 ng/ml. At this concentration the signal-to-noise ratio was 3. In addition, the response was significantly different from blank plasma ($P=0.002$). At 2.5 ng/ml the percent deviation from the nominal concentration and the relative standard deviation were both less than 20%. Thus, 2.5 ng/ml was defined to be the LLQ. At all other concentrations up to the ULQ (4000 ng/ml) the percent deviation from the nominal concentration and the relative standard deviation were less than 15%. A typical chromatogram of a spiked plasma sample of 40 ng/ml is shown in Fig. 2D.

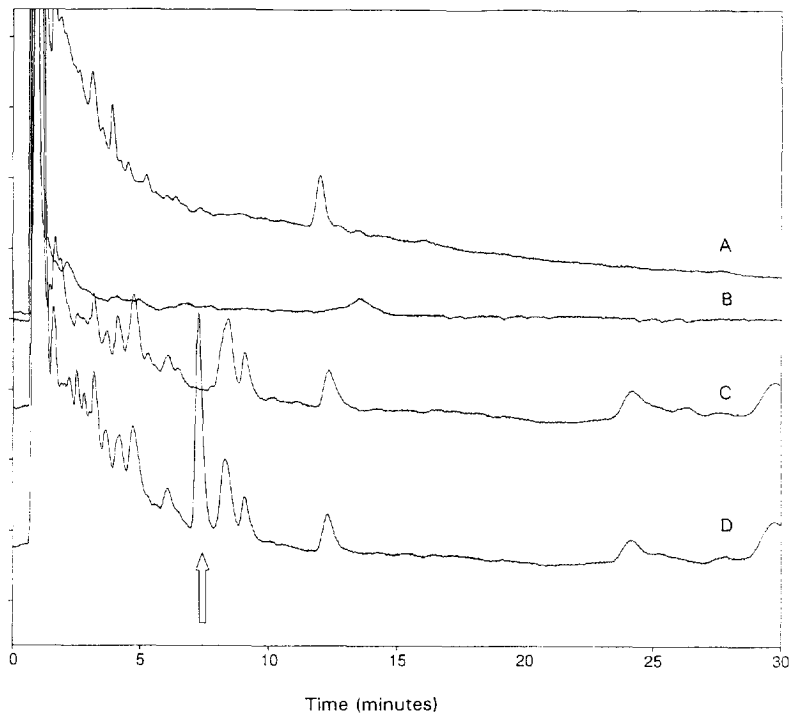

Fig. 2. Chromatograms of blank saliva (A), blank cerebrospinal fluid (B), blank plasma (C), and a spiked plasma sample of 40 ng/ml saquinavir (D). The saquinavir peak is indicated by an arrow (D).

Table 2
Accuracy and precision for the analysis of saquinavir in human plasma

Concentration (ng/ml)	Accuracy (%)	C.V. (%)	Precision (%)		n
			Between-day	Within-day	
88	109	3.6	3.9	3.9	9
881	102	3.3	3.4	3.4	9
1537	104	4.8	5.2	5.2	9

C.V., coefficient of variation; *n*, number of replicates.

3.5. Validation: accuracy, precision, linearity and stability

The results from the validation of the method in human plasma are listed in Table 2. The use of the peak area in combination with a weight factor of (1/conc.) resulted in a minimal deviation from nominal concentrations. The method proved to be accurate (average accuracy at three concentrations 102–109% of the real concentrations) and precise (within-day precision ranged from 3.4 to 5.2%). No significant additional variation was observed as a result of performing the assay on different days. Correlation coefficients (r^2) of calibration curves were >0.99 as determined by least-squares analysis.

All calibration curves proved to be linear in the range of 2.5–4000 ng/ml with use of the *F*-test for lack of fit as an indicator of linearity of the regression model.

The stability of saquinavir at various conditions is shown in Table 3. Stability of saquinavir when stored for 1 h at 60°C could not be determined because of repeated coagulation of the samples. Under all other conditions tested saquinavir is stable with concentrations of at least 91% of the initial concentration.

3.6. Analysis of patient samples

The applicability of the assay for pharmacokinetic research in HIV-1-infected patients was demonstrated. Plasma pharmacokinetics of saquinavir showed large interindividual variation. This has recently also been reported by other investigators [8]. This large interindividual variation may be caused by low (4%) and variable oral bioavailability of the currently used hard capsule formulation of saquinavir, and/or by extensive cytochrome P450 metabolism of this drug [9]. Indications of a pharmacokinetic–pharmacodynamic relationships have been reported, suggesting enhanced antiviral efficacy with increased exposure to saquinavir [1,3,9]. Thus, monitoring saquinavir pharmacokinetics may be imperative to ensure optimal drug efficacy and to prevent the risk of drug resistance in individual patients.

In six patients, concentrations of saquinavir in saliva have been determined and were found to be below 10 ng/ml (range, <2.5–8.5 ng/ml). These low concentrations may be the result of high binding of saquinavir to plasma proteins (>98%) [10].

The plasma concentration–time profile in a patient after ingestion of 600 mg of saquinavir as determined by the currently described bioanalytical method is shown in Fig. 3.

In conclusion, a validated assay for the quantitative determination of saquinavir in human plasma, saliva, and cerebrospinal fluid samples has been

Table 3
Stability of saquinavir in spiked human plasma samples

Storage conditions	Concentration (ng/ml)	Recovery (%)	C.V. (%)	n
24 h at 25°C	10.1	97	7.5	4
	101	93	0.8	4
	1010	92	1.0	4
7 days at 4°C	10.1	98	6.2	4
	101	95	2.0	4
	1010	99	5.7	4
30 days at −30°C	10.1	95	1.6	4
	101	93	0.8	4
	1010	91	1.9	4

C.V., coefficient of variation; *n*, number of replicates.

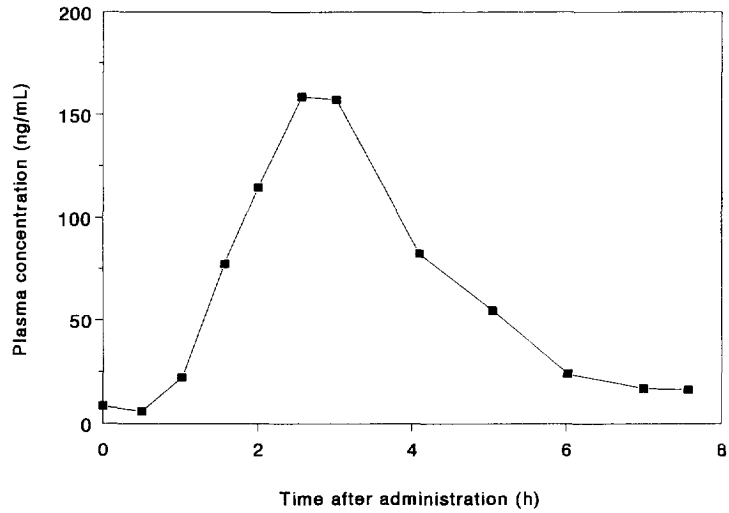

Fig. 3. Plasma concentration versus time curve of saquinavir after oral administration with breakfast of 600 mg to an HIV-1-infected patient (chronic use).

described. The assay meets the current requirements as to the validation of a bioanalytical methodology and can be used for pharmacokinetic studies with saquinavir in HIV-infected patients. The currently described HPLC assay can readily be used in a hospital laboratory for the monitoring of saquinavir concentrations.

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