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Highly sensitive determination of saquinavir in biological samples using liquid chromatography–tandem mass spectrometry

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Abstract

A selective and sensitive method for the determination of the HIV protease inhibitor saquinavir in human plasma, saliva, and urine using liquid–liquid extraction and LC–MS–MS has been developed, validated, and applied to samples of a healthy individual. After extraction with ethyl acetate, sample extracts were chromatographed isocratically within 5 min on Kromasil RP-18. The drug was detected with tandem mass spectrometry in the selected reaction monitoring mode using an electrospray ion source and $^{2}H_{5}$-saquinavir as internal standard. The limit of quantification was 0.05 ng/mL. The accuracy of the method varied between −1 and +10% (SD within-batch) and the precision ranged from +4 to +10% (SD batch-to-batch). The method is linear at least within 0.05 and 87.6 ng/mL. After a regular oral dose (600 mg) saquinavir concentrations were detectable for 48 h in plasma and were well correlated with saliva concentrations (r^2=0.9348, mean saliva/plasma ratio 1:15.1). The method is well suited for low saquinavir concentrations in different matrices.

Keywords: Saquinavir

1. Introduction

Saquinavir was the first antiretroviral drug from the group of human immunodeficiency virus (HIV) protease inhibitors, including amprenavir, indinavir, lopinavir, nelfinavir, and ritonavir, which have markedly improved morbidity and mortality in HIV-infected patients [1]. It is a competitive inhibitor of the HIV-1 and HIV-2 protease-mediated cleavage of the HIV gag and pol polypeptides and is used in combination with other protease inhibitors, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors [2,3]. The development of methods for the determination of plasma concentrations of saquinavir and other protease inhibitors is important to help to detect and prevent impending treatment failure in HIV therapy (e.g. due to resistance development at sub-therapeutic concentrations [4]) [5–7]. On the other hand, the bioavailability of saquinavir is very low and there is a need to develop strategies to increase saquinavir plasma concentrations [7–9]. For both cases, analytical methods depending on high-pressure liquid

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chromatography (HPLC) coupled to ultraviolet (UV) detection have been described. Especially for saquinavir, two HPLC–UV methods were developed with a limit of detection close to 1 ng/mL plasma [10,11] and a few further publications showed the quantification of saquinavir beside other protease inhibitors in double or triple therapy with higher limits of quantification [12–15]. In addition, saquinavir was also quantified in combination with the non-nucleoside reverse transcriptase inhibitors delavirdine and efavirenz [16,17]. Alternative methods, such as immunoassay and liquid chromatography–tandem mass spectrometry (LC–MS–MS), for the determination of saquinavir have been developed [18]. The HPLC–UV assay [limit of quantification (LOQ) 1 ng/mL] was compared with a radioimmunoassay (LOQ 0.5–1.0 ng/mL) and validated against an LC–MS–MS assay (LOQ 0.4 ng/mL) [19]. The latter used solid-phase extraction (SPE) coupled to a short LC column and a tandem mass spectrometer using positive-ion atmospheric pressure chemical ionisation (APCI) in the selected reaction monitoring mode (SRM). The assay permitted rapid quantification over the range 0.4–200 ng/mL using a stable isotopically labelled form of saquinavir as internal standard.

The determination of protease inhibitor concentrations is essential for studies assessing the frequent food–drug and drug–drug interactions of this group of therapeutics. The pharmacokinetics of saquinavir are characterised by restricted drug absorption, excessive protein binding and a large volume of distribution. The effectiveness of saquinavir strongly depends on the accessibility of the target structures infected with the virus [20] and low concentrations are associated with treatment failure [6,20,21]. Monitoring of its concentration in different body compartments will aid in the understanding of its pharmacological effects and the circumstances leading to treatment failure. Thus far no published method for saquinavir determination in urine is available. Moreover, non-invasive saliva measurements, which are frequently used for therapeutic drug monitoring of other compounds [22–24], have not been evaluated, likely because of the absence of assays with sufficient sensitivity.

This paper describes a new rapid method for the quantification of low saquinavir concentrations in plasma, urine, and saliva samples and its successful application to a series of samples of a healthy volunteer. The method was additionally applied in a study including eight healthy individuals in whom the bioavailability of saquinavir was increased by the pharmaceutical aid cremophor EL [25]. The high sensitivity of this method was a prerequisite for the successful conclusion of this latter study in which >1000 biological samples were assayed. In contrast to all previously published methods this method is at least six-fold more sensitive than the most sensitive method [19] and can be applied to different biological matrices. It may therefore be suitable for therapeutic drug monitoring of saquinavir concentrations, which may be a way of detecting non-compliance and quantifying individual pharmacokinetics as a basis for sophisticated and personalised dose optimisation strategies [21], a concept which awaits confirmation in prospective clinical trials.

2. Subject, materials and methods

2.1. Volunteer

One healthy, male, non-smoking individual participated after having given written informed consent. The individual was found to be healthy by medical history, physical examination, laboratory screening including haematological and biochemical blood tests, and a 12-lead electrocardiogram.

2.2. Clinical study design

The study protocol was approved by the Ethics Committee of the Medical Faculty of the University of Heidelberg and was conducted at the Department of Internal Medicine VI, Clinical Pharmacology and Pharmacoepidemiology in accordance with GCP, the Declaration of Helsinki and subsequent amendments.

The individual fasted for 12 h prior to and until 4 h after administration of the study drug. Food and beverages containing alcohol or methylxanthines were not allowed from 12 h before drug administration until the last blood sample was drawn. The study drug was administered with 200 mL of water and consisted of 600 mg Invirase (saquinavir).

Standardised liquid food (Fresubin) was served 4 (750 mL) and 8 h (500 mL) after administration. Blood samples (7.5 mL) were drawn immediately before and 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0, 12.0, 24.0, and 48.0 h after dosing. The samples were immediately centrifuged (3000 *g* for 15 min at 4 °C) and the plasma was put on ice within 30 min. Saliva samples (≥1 mL; stimulated by chewing parafilm ''M'', American National Can, Greenwich, USA [22]) were collected up to 12 h at the same sampling times by direct collection into Falcon tubes. Urine was collected in four fractions (0, 0–12, 12–24 and 24–48 h). The volumes of the urine fractions were measured and urine aliquots and all other samples were stored at −20 °C until analysis.

2.3. Materials

Invirase capsules (containing 200 mg saquinavir) were purchased from Hoffmann-La Roche (Grenzach-Wyhlen, Germany). Saquinavir (Ro 31-8959/008) and $^{2}H_{5}$-saquinavir (Ro 31-8959/048) were generous gifts from Roche Products (Hertfordshire, UK) (Fig. 1). All reagents and solvents used for the chromatographic, spectroscopic, and sample procedures were of analytical or higher quality and were purchased from Fluka (Neu-Ulm, Germany), E. Merck (Darmstadt, Germany), and Promochem (Wesel, Germany). The water was deionised and filtered by an HP 6UV/UF TKA system (TKA, Niederelbert, Germany).

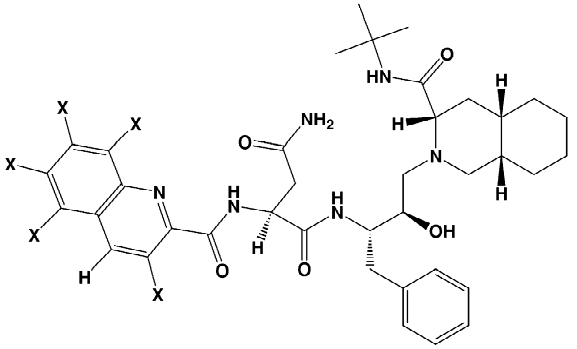

Fig. 1. Chemical structures and codes of saquinavir and $^{2}H_{5}$-saquinavir.

2.4. Standard solutions, calibration and quality control (QC) samples

2.4.1. Internal standard (I.S.)

Ro 31-8959/048 ($^{2}H_{5}$-saquinavir-mesylate, 6.26 mg) was weighed into a volumetric flask (50 mL) and filled up to volume with acetonitrile–water (1:1). From this stock solution, an I.S. subsolution (I.S.-1) containing 503 ng $^{2}H_{5}$-saquinavir/mL was prepared.

2.4.2. Calibration standard (Cal)

Ro 31-8959/008 (saquinavir-mesylate, 5.0 mg) was weighed into a volumetric flask (50 mL) and filled up to volume with acetonitrile–water (1:1). Calibration subsolutions (Cal-1 to -3) were prepared and contained the following amounts of pure saquinavir: 933 (Cal-1), 94 (Cal-2) and 9.4 ng/mL (Cal-3).

2.4.3. Quality control standard

Ro 31-8959/008 (saquinavir-mesylate, 4.1 mg) was weighed into a volumetric flask (50 mL) and filled up to volume with acetonitrile–water (1:1). Calibration subsolutions were prepared according to the calibration standards resulting in the following amounts: 906 (QC-1), 80.5 (QC-2) and 8.05 ng/mL (QC-3).

2.4.4. Calibration samples

Blank samples (1 mL) were spiked with I.S. subsolution (I.S.-1, 10 μL) and calibration subsolutions (Cal-1 to Cal-3), resulting in nine calibration samples (Cal-A to Cal-I) representing concentrations 0, 0.05, 0.09, 0.45, 0.88, 4.45, 8.76, 44.5, and 87.6 ng/mL. The calibration samples were further processed as for the study samples.

2.4.5. Quality control samples

QC samples at three different concentrations were prepared by spiking blank plasma (1 mL) with internal standard (I.S.-1, 10 μL) and the respective calibration subsolutions (QC-1 to QC-3). The resulting concentrations were 0.07 (QC-A), 4.08 (QC-B) and 90.9 ng/mL (QC-C). Within each batch of

study samples, six QC samples (QC-A to QC-C in duplicate preparation) were prepared. The quality controls were further processed as for the study samples.

2.5. Sample preparation

Before liquid–liquid extraction, the sample (1 mL plasma, saliva, or urine) was spiked with $^{2}H_{5}$-saquinavir (10 μL I.S. subsolution I.S.-1), resulting in 5 ng $^{2}H_{5}$-saquinavir/mL sample. Subsequently, the spiked sample was alkalinised with 2 m*M* K_3PO_4 (400 μL), ethyl acetate (5 mL) was added and shaken automatically (15 min). After centrifugation at 2000 *g* (10 min) the organic layer was separated and evaporated to dryness via a gentle stream of nitrogen at 40 °C. Reconstitution of the extracts was done by addition of eluent B (300 μL) and eluent A (200 μL). The instrumental analysis of reconstituted extracts was performed within 8 h.

To differentiate between free saquinavir and conjugates of saquinavir in urine, urine samples were additionally hydrolysed before extraction by adding 400 μL 1 *M* NaOH solution to 1 mL urine after spiking with the internal standard. The samples were incubated for 1 h at 50 °C and subsequently extracted and handled according to the above-mentioned procedure.

2.6. Instrumental analysis parameters

The HPLC system (Thermo Finnigan/TSP) consisted of a quaternary LC pump (Model P4000) with degasser and a sample cooling (15 °C) autosampler (Model AS3000) with integrated column heater. The injection volume was 10 μL. For isocratic chromatographic separation at 40 °C a Kromasil C_{18} column 100 A 3 μm, 70×2 mm I.D. with integrated guard column was used. The eluent (55% A/45% B) consisted of 0.1% (by volume) aqueous acetic acid including 5 m*M* ammonium acetate (A) and acetonitrile (B). The flow-rate was 0.35 ml/min and was introduced without splitting into the electrospray ion source (ESI) of a triple-stage quadrupole mass spectrometer (Thermo Finnigan TSQ 7000 with API-2 ion source and performance kit). ESI interface parameters were as follows: middle position, spray voltage 4.5 kV, sheath gas (N_2) 60 p.s.i., aux gas (N_2) 20 scales, capillary heater temperature 375 °C. The mass spectrometer was automatically tuned using a mixture of polypropylene glycols and Xcalibur software guide procedures. The voltages responsible for the spray focus (heated capillary, skimmer lens, lens L11) were optimised. For this purpose an aqueous solution of pure saquinavir (0.1 mg/mL) was introduced continuously into the LC eluent via a syringe pump (1 μL/min) and the intensity of the $[M+H]^+$ ion (*m*/*z* 671.4 saquinavir) was monitored and adjusted to maximum. In the full-scan MS mode, 1.20 kV multiplier voltage was chosen. SRM measurements were performed at 1.55 kV multiplier voltage. MS–MS transitions monitored in the positive ion mode were *m*/*z* 671.4→*m*/*z* 570.3 for saquinavir and *m*/*z* 676.4→*m*/*z* 575.3 for $^{2}H_{5}$-saquinavir. The parameters influencing these transitions were optimised: the Ar pressure in the collision quadrupole q2 was set to 3.0 mbar, the offset voltage on q2 was adjusted to 36 V and the resolution on the parent quadrupole Q1 was slightly decreased.

The APCI ion source was used in the full-scan MS mode for comparison of the ion yield with the ESI ion source. The following APCI parameters were used: vaporiser temperature 500 °C, charge on corona needle 5 μA, heated capillary 350 °C, sheath gas (N_2) 40 p.s.i., aux gas (N_2) 20 scales.

2.7. Evaluation of the analytical method

Analytical method validation was performed in accordance with the recommendations published by the US Food and Drug Administration (FDA) [26]. Accuracy was calculated on the basis of the difference between the averaged measurements and the nominal value, and expressed in percent. Precision was defined as the ratio of the standard deviation and the mean calculated value in percent. These values are given within-batch and batch-to-batch.

For this purpose, analytical batches ($n=3$) each containing nine calibration samples, 18 quality control samples at three different concentrations and different numbers of blank plasma were analysed. From these values the accuracy and precision of the method were calculated.

Further analytical batches ($n=10$) with study

samples from a recently published study [25] included nine calibration samples, six quality control samples at three different concentrations and different numbers of blank samples and resulted in additional data on batch-to-batch accuracy and precision. Data are expressed as mean values±standard deviation (SD).

3. Results and discussion

3.1. Sample preparation

Liquid–liquid extraction with ethyl acetate was chosen for a fast and easy sample preparation. The dried residue from 1 mL samples was reconstituted with 500 μL eluent volume. Further decrease of the eluent volume resulted in lower extraction recoveries, which was caused by sorption effects to a solid precipitation built up with low eluent volumes. Reconstitution with 500 μL eluent gave recoveries from plasma of 84.5% at 3.58 ng/mL and 89.1% at 71.8 ng/mL. Urine samples were extracted after treatment with and without sodium hydroxide. In separate experiments this procedure involving strong alkali (pH 13) was found not to degrade saquinavir, which was shown by comparing alkaline extractions of spiked blank samples (calibration and QC samples) with untreated samples.

3.2. Mass spectra, fragmentation and SRM parameters of saquinavir

In a first step the saquinavir response to ESI and APCI was evaluated by recording direct inlet full-scan mass spectra in the positive and negative ionisation mode with both sources (introduction of saquinavir via a syringe pump in the LC eluent). In the negative ion mode the signal intensities obtained with both sources were too low for quantitative measurements. In the positive mode, ESI spectra revealed 20-fold higher signals for m/z 671 ($[\text{saquinavir}+\text{H}]^+$) compared to APCI. This behaviour was expected because saquinavir is an alkaline compound containing a hydroxyl group and six nitrogens in different chemical environments (amide, secondary and tertiary amine, peptide bondings; see Fig. 1). Further assay development was therefore limited to the ESI source. This step might represent the main cause for the marked improvement in sensitivity in comparison to Ref. [19], where the APCI ion source was used. The collision-induced decomposition (CID) of protonated saquinavir (m/z 671.4, $[\text{M}+\text{H}]^+$) results in the loss of the *N-tert.*-butyl carboxamide group, leading to the main daughter fragment m/z 570.3 ($[\text{M}-100]^+$). This is also the main fragmentation pathway when the APCI ion source is used. Other fragmentations showed relative intensities below 30% and are not likely to improve the sensitivity when used in selected reaction monitoring (Fig. 2). Additional tuning of the ESI source and CID parameters onto the transition m/z 671.4→m/z 570.3 (saquinavir) and m/z 676.4→m/z 575.3 ($^2\text{H}_5$-saquinavir) further improved the sensitivity.

3.3. Performance and validation of the analytical method

The HPLC parameters were optimised for the fast detection of saquinavir with mass spectrometry by choosing a short and narrow reversed-phase HPLC column. The solvent system, which contained only volatile compounds, was reduced to the specific requirements of the ESI source. Fig. 3 shows selected blank and spiked blank chromatograms as well as a real plasma sample: the amount of acetonitrile in the eluent was adjusted for elution of saquinavir within 3 min. These parameters resulted in short chromatograms (5 min) with peak half widths in a maximum of 30 s. Using selective and sensitive tandem mass spectrometric detection, no matrix interference was observed in the blank plasma of six different individuals. In general, a co-eluting matrix is able to influence the ionisation process and to contaminate the heated capillary, resulting in greater variation, particularly at the LOQ level. The solvent system was optimised to separate saquinavir (R_t at about 2 min) from the main matrix, which eluted at 0.5 min. The LOQ was 0.05 ng/mL with an accuracy within 20%. The signal-to-noise ratio at LOQ was at least 5:1. Without cleaning the heated capillary, more than 100 measurements were possible. Regarding the extraction recovery and subsequent measurement of the QC-A sample at 0.07 ng/mL, the total

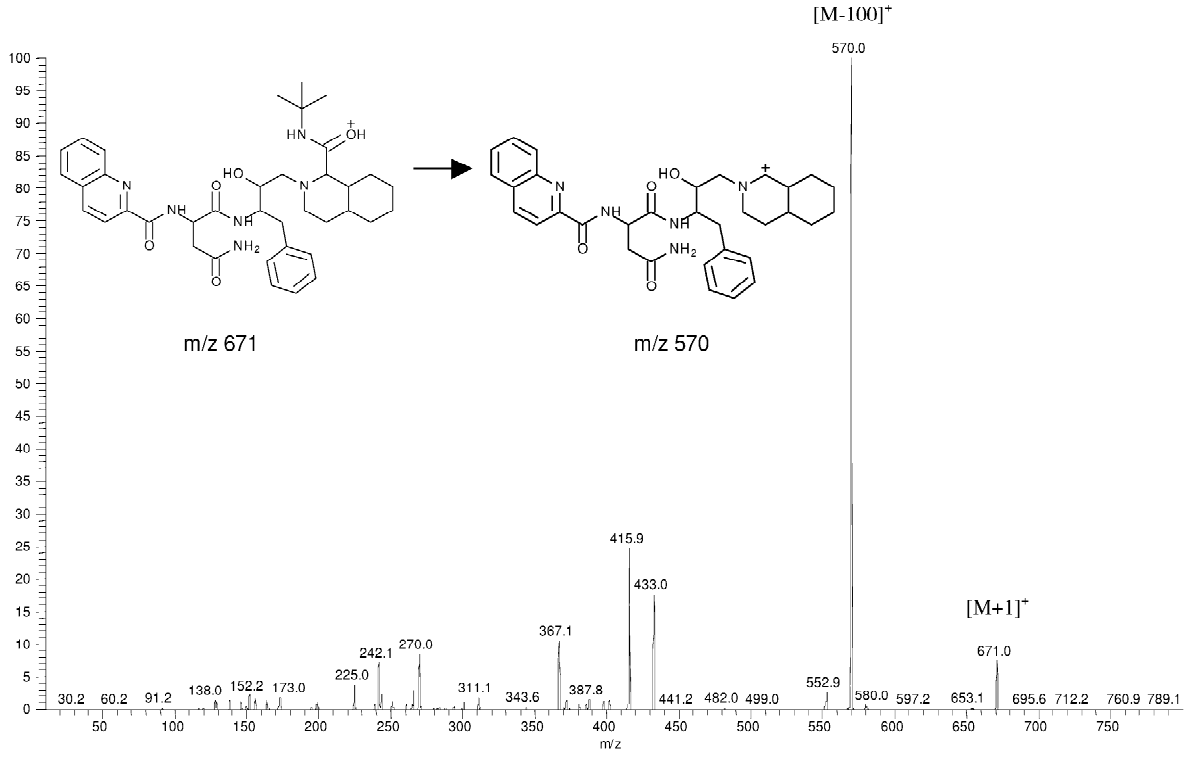

Fig. 2. Tandem mass spectrum (daughter ion scan) of the saquinavir parent ion m/z 671 performed with ESI and CID at 36 V using Ar at 3.0 mbar and the proposed fragmentation pathway according to Mass Frontier 2.0 (Thermo Finnigan).

amount injected onto the LC–MS–MS system was 1.1 pg. This excellent sensitivity was a prerequisite to analyse samples from a bioavailability study using this procedure [25].

The analytical method validation was performed with three analytical batches according to Ref. [26] and gave the following results: accuracy within-batch was 9.5% standard deviation (SD) at QC-A, 2.3% SD at QC-B, and 0.1% SD at QC-C. Accuracy batch-to-batch was 11.1% SD at QC-A, 6.7% SD at QC-B, and 0.3% SD at QC-C. The mean precision ($n=18$) was 10.0% SD (-2.8 to $+19.7\%$) at QC-A, -3.3% SD (-7.5 to $+3.1\%$) at QC-B and -1.0% SD (-7.1 to $+5.0\%$) at QC-C. Table 1 shows the results of the batch-to-batch accuracy during a bioavailability study [25] with 10 analytical batches. The correlation coefficients (r^2) from the calibration curves of study batches were at least 0.9950 or higher.

3.4. Application of the method to samples of a single volunteer

In HIV-infected patients, saquinavir (Invirase or Fortovase) is usually co-administered with ritonavir or nelfinavir to increase saquinavir bioavailability through inhibition of cytochrome P450 3A4, which is the major isozyme responsible for the metabolic clearance of saquinavir. Under these conditions, plasma concentrations of patients are in the range of about 200 to 1800 ng/mL [6] and can be routinely analysed by HPLC–UV. Significantly lower plasma concentrations will be reached when saquinavir is administered alone. Hence more sensitive methods are required when the effect of food components [8] or pharmaceutical aids to enhance saquinavir bioavailability [25] is evaluated or if tissue distribution or elimination into further biological compartments are of interest (e.g. urine, saliva, free fraction in

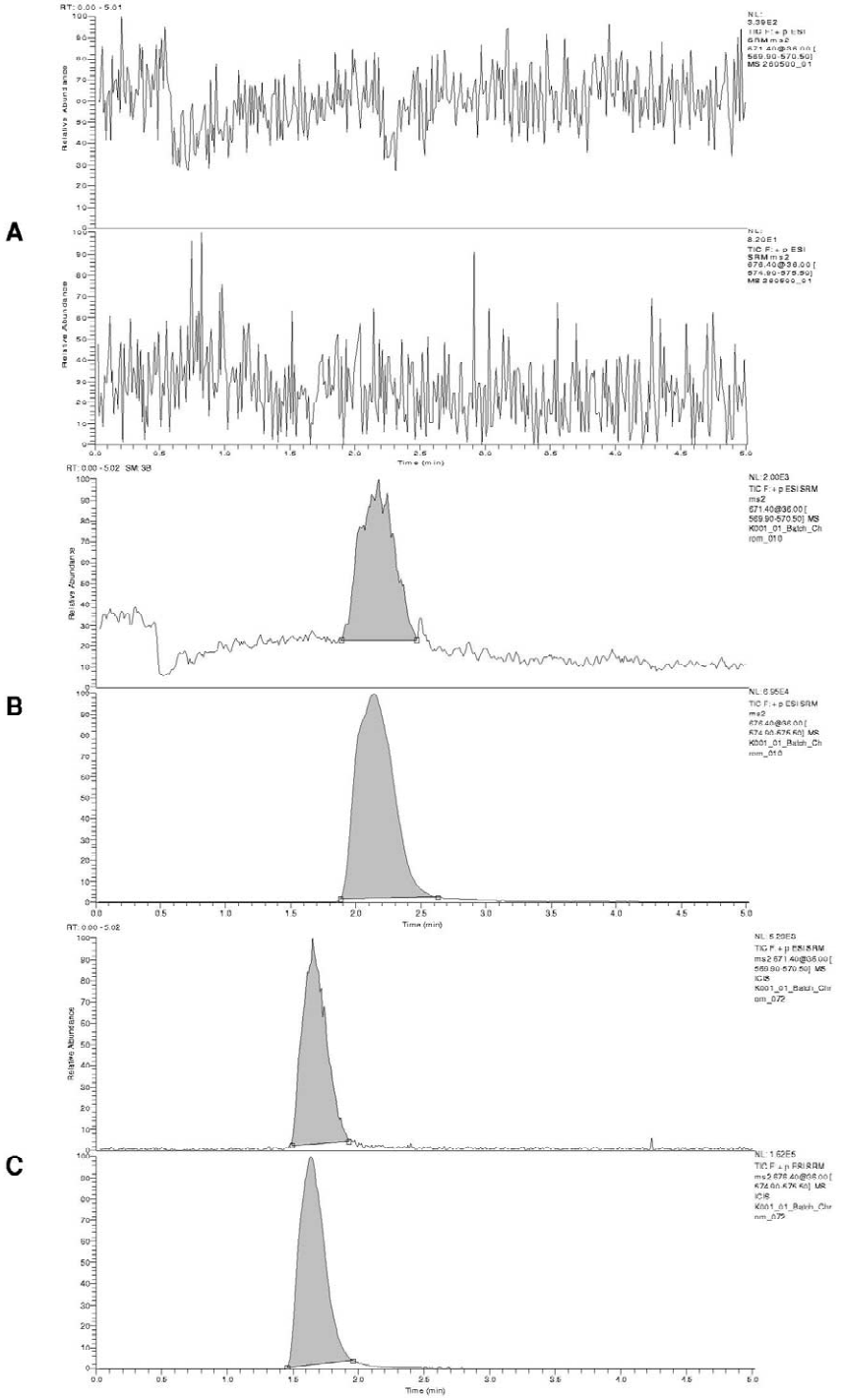

Fig. 3. LC–MS–MS chromatograms of extracted plasma samples spiked with internal standard $^{2}H_{5}$-saquinavir. Upper traces: saquinavir 671.4→570.2, CID 36 V, Ar 3.0 mbar. Lower traces: $^{2}H_{5}$-saquinavir-trace 676.4→575.2, CID 36 V, Ar 3.0 mbar. (A) Blank plasma sample. (B) Quality control sample at QC-A concentration (0.07 ng/mL). (C) Plasma sample 48 h (calculated concentration 0.17 ng/mL).

Table 1
Results of analytical method validation: batch-to-batch accuracy ($n = 10$ batches)

	QC-A	QC-B	QC-C
Adjusted concentration (ng/mL)	0.07	4.08	90.9
Recalculated mean±SD (ng/mL)	0.07±0.01	4.12±0.15	89.6±3.96
Accuracy (%)	+10	+1	−1
Precision C.V. (%)	10	4	4

plasma). This method gave the opportunity to quantify saquinavir in plasma samples from bioavailability studies at least for 48 h after oral administration of a single regular dose of 600 mg Invirase.

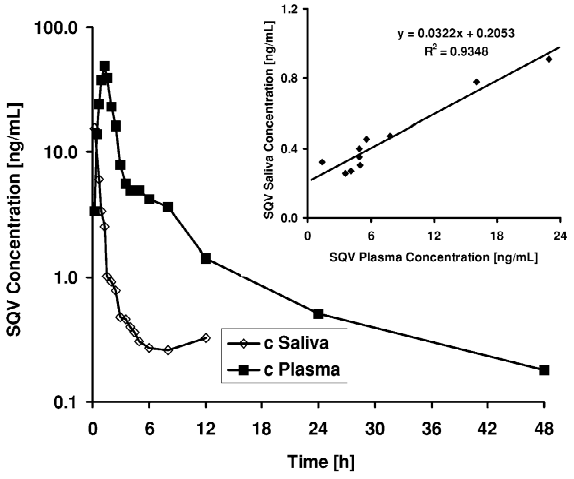

Fig. 4. Concentration–time profile of saquinavir in plasma and saliva of a healthy individual after oral administration of 600 mg Invirase. Insert shows the correlation between plasma and saliva concentrations between 2 and 12 h.

After 48 h, plasma concentrations varied from 0.06 to 0.58 ng/mL (mean 0.20 ng/mL, $n = 32$). This covers about four to six half-lives, resulting in accurate determination of its pharmacokinetics [25]. In this study the plasma concentrations of a healthy individual ranged from 0.18 to 48.4 ng/mL (Fig. 4). These data are roughly comparable to those observed in an earlier bioavailability study in which saquinavir was also administered to fasted volunteers [8].

Additionally, urine was analysed in this study, which is essential when drug–drug interactions and the influence of co-administered drugs on renal clearance are evaluated. Due to the low bioavailability of saquinavir (1% in fasted and 4% in fed state [2,8]) and its substantial hepatic degradation with subsequent elimination into the bile, the urinary concentrations, absolute amounts in urine, and elimination rate via the kidneys are low (Table 2). In total, less than 0.2% of the administered saquinavir was eliminated via urine, which is approximately 20% of the estimated amount of drug absorbed. As expected, comparison of the results with and without alkaline hydrolysis revealed no significant conjugation of saquinavir.

Table 2
Results of the urine saquinavir analysis: concentration in urine fractions, amount eliminated, and elimination rate in percent of administered saquinavir

Time (h)	Saquinavir concentration (ng/mL)		Saquinavir eliminated (μg)		Saquinavir eliminated (%)	
	Without[a]	With[a]	Without	With	Without	With
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0–12	229.8	251.4	826.1	903.5	0.138	0.151
12–24	35.31	44.55	78.27	98.76	0.013	0.016
24–48	11.17	13.09	12.49	14.64	0.002	0.002

n.d., not detectable (<LOQ).
[a] Without/with hydrolysis.

The high sensitivity of this assay was a prerequisite to study saliva concentrations. Salivary drug concentrations are influenced by many factors, with salivary pH, fractional contribution of the different salivary glands to total saliva, and the stimulation procedure being among the most critical. Thus far the best correlations with plasma drug concentrations were found when concentrations were determined in stimulated saliva [22–24]. Stimulation is often achieved by mastication while chewing cotton rolls or plastics. For instance, a good correlation of saliva and plasma concentrations of nevirapine, a non-nucleoside reverse transcriptase inhibitor, was reported for stimulated saliva [24]. We therefore attempted to maximise flow immediately before saliva collection and decided to stimulate flow by letting the participant chew inert parafilm. The saliva saquinavir concentrations were found to be roughly one order of magnitude lower than the corresponding total plasma concentrations (mean 8.1±5.5% SD) and the mean saliva/plasma ratio was 1:15.1. In general, as a non-invasive procedure, saliva monitoring has several important advantages because it can be repeated unrestrictedly and may reflect free (=active) plasma concentrations more closely than total concentrations. Especially the free plasma concentrations of saquinavir are particularly low due to extensive binding to plasma proteins of about 99%. The availability of a sensitive assay is therefore a prerequisite for the evaluation of saliva and free drug monitoring in saquinavir-treated patients, including children in whom monitoring may be essential because of the substantial pharmacokinetic differences as compared to adults [27]. The data obtained in our study participant suggest that saliva concentrations are possibly a marker for total plasma concentrations. The correlation of plasma with saliva concentrations (insert Fig. 4) is highly significant, when comparing the data from 2 to 12 h after administration. Before 2 h the saliva samples appeared to be contaminated by drug residues originating from the preceding oral drug administration. This assumption is further supported by the fact that saliva concentrations early after dosing (0.25 and 0.75 h) were similar to or even exceeded the corresponding plasma concentrations. This is a key problem of comparing saliva and plasma concentrations, because—unless given through a feeding tube—drugs may be sorbed to the mucous membrane during deglutition. Hence, using this method, the meaning and clinical usefulness of saquinavir monitoring in saliva can be evaluated. Therefore, more samples from patients receiving saquinavir are necessary. Hence, prospective studies in patients have now to be performed which should involve steady-state conditions, regular (i.e. higher) saquinavir doses, and also assess the potential effects of co-medication.

4. Conclusion

This LC–MS–MS assay combined with liquid–liquid extraction and a deuterated internal standard is highly sensitive, precise, and fast for the determination of saquinavir in biological samples from bioavailability studies. The method is calibrated and validated in the concentration range of therapeutic interest even when saquinavir is administered without a cytochrome P450 enzyme inhibitor. The limit of quantification allows plasma drug monitoring for at least 48 h and the practicability of the procedure was confirmed with more than 1000 authentic plasma samples within a pharmacokinetic study [25]. Additionally, the sensitivity of this method allows the measurement of saquinavir in further biological compartments where saquinavir concentrations are low or where the compound is mainly present in unbound form (e.g. saliva and urine).

Acknowledgements

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