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Determination of saquinavir in human plasma by high-performance liquid chromatography

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Abstract

We developed and characterized a high-performance liquid chromatography (HPLC) assay for the determination of saquinavir, an HIV protease inhibitor, in human plasma samples. Extraction of plasma samples with diethyl ether resulted in quantitative recovery of both saquinavir and its stereoisomer Ro 31-8533 which was used as an internal standard. The assay was performed isocratically using 5 m*M* H_2SO_4 (pH 3.5) and acetonitrile (75.5:24.5, v/v) containing 10 m*M* tetrabutylammonium hydrogen sulfate (TBA) as a mobile phase, a Nucleosil 3C8 column kept at 45°C and UV detection at 240 nm. Using this method, saquinavir and Ro 31-8533 can be separated from endogenous substances, and in the concentration range of 5–110 ng/ml the relative standard deviations for the determination of saquinavir were below 5%. The detection limit of saquinavir in human plasma was 1 ng/ml. The usefulness of the method was demonstrated by quantification of saquinavir in plasma of human subjects treated with 600 mg of saquinavir per os or 12 mg intravenously. © 1997 Elsevier Science B.V.

Keywords: Saquinavir

1. Introduction

Saquinavir is a competitive inhibitor of the HIV-1 and HIV-2 protease-mediated cleavage of the HIV *gag* and *pol* polypeptides, and was the first member of this class of drugs which became commercially available for the treatment of patients with HIV infection [1]. Saquinavir has been developed using computer-assisted design techniques and mimics the transition state of the phenylalanine–proline cleavage site in HIV polypetides [2]. The pharmacokinetics of saquinavir are characterized by a low bioavailability (approximately 4% when ingested after a meal and lower when taken in fasted state) due to extensive first-pass metabolism, a large volume of distribution (steady state volume of distribution approximately 700 l), suggesting considerable tissue-binding, and a variable elimination half-life equaling approximately 13 h for the terminal elimination phase in healthy volunteers [1,3]. In order to improve the low bioavailability of saquinavir, clinical studies will be neces-

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sary to investigate different galenic formulations and the pharmacokinetic interaction with inhibitors of cytochrome P450 3A4 (CYP3A4), the main isozyme responsible for saquinavir metabolism [1,4]. To perform these studies, a fast and reliable assay for the quantification of saquinavir in human plasma sample is needed.

We therefore developed a high-performance liquid chromatography (HPLC) method which allows rapid and accurate determination of saquinavir in human plasma samples.

2. Experimental

2.1. Reference compounds

The reference substances saquinavir mesylate Ro 31-8959 (*cis-N-tert*-butyl-decahydro-2-[2(*R*)-hydroxy-4-phenyl-3(*S*)-[[(*N*-2-quinolylcarbonyl)-L-asparaginyl]amino]butyl] - (4a*S*,8a*S*) - isoquinoline - 3(*S*)-carboxamide methylsulphonate) and internal standard Ro 31-9533, which is a stereoisomer of saquinavir, were kindly supplied by Hoffman-La Roche (Basel, Switzerland).

2.2. Chemicals

Diethyl ether, acetonitrile of HPLC grade, sulfuric acid and $K_3PO_4 \cdot 3H_2O$ were obtained from E. Merck (Darmstadt, Germany). Tetrabutylammonium hydrogen sulfate (TBA) was obtained from Sigma (Buchs, Switzerland).

2.3. Standards

Saquinavir mesylate equivalent to 5 mg of saquinavir base were dissolved in 20 ml of methanol. Drug-free human heparinized plasma (10 ml) was spiked with this methanolic solution to obtain a stock sample containing 1 μg/ml. Plasma standards were then prepared by diluting the stock sample with human plasma to give final concentrations of saquinavir base in the range of 2.5–100 ng/ml. Stored in aliquots of 2 ml at −20°C, the plasma standards are stable for at least two months. The internal standard for the HPLC assay was prepared by dissolving 1 mg Ro 31-9533 in 10 ml of methanol and diluting with the same solvent to 250 ng/ml. The solutions were stored at 4°C for one month.

2.4. Instrument parameters

The HPLC system consisted of two pumps LKB 2150, a controller LKB 2152, a column oven LKB 2155 and an autosampler LKB 2157 (Bromma, Sweden); Saquinavir and its stereoisomer Ro 31-9533 were separated on a Nucleosil HPLC column (120-3C8 125×3 mm; Macherey-Nagel, Oensingen, Switzerland) protected by a guard column (15×3 mm) packed with the same material; the columns were maintained at 45°C. The standard mobile phase consisted of 5 m*M* sulfuric acid and acetonitrile (75.5:24.5, v/v) containing 10 m*M* TBA pH 3.5. A flow-rate of 1 ml/min was maintained through the column (generated pressure 200 bar). Saquinavir and internal standard were detected by measuring the absorption at 240 nm by an UV detector Spectroflow 773 (Kratos, Westwood, NJ, USA) and the signals were computerized by the Nelson data management program (Cupertino, CA, USA).

2.5. Extraction procedure

The drug concentration of unknown samples was estimated by rapid screening using 0.2 ml plasma and the samples were diluted with drug free plasma to a concentration <100 ng/ml. Saquinavir standard or unknown samples (1 ml) and 0.1 ml of internal standard Ro 31-9533 solution (equivalent to 25 ng) were then dispensed into 15-ml glass test tubes. A 100-μl volume of 2 *M* K_3PO_4 and 3 ml *n*-hexane were added. The tubes were capped and mixed on a reciprocating shaker (Labshaker, Basel, Switzerland) at 300 rpm for 5 min. After centrifugation at 1500 *g* for 5 min, the organic phase of the washing step was discarded by aspiration. Saquinavir and its related compound(s) were then extracted twice with 5 ml of diethyl ether by shaking at 300 rpm for 10 min each. The respective organic phases were separated by centrifugation, pooled and evaporated to dryness at 40°C under a stream of air. The residue was dissolved in 75 μl of mobile phase and 50 μl of the aliquot were injected onto the HPLC column. On every working day, six standard samples (2.5, 5, 10, 20, 50, 100 ng/ml) were extracted. The peak height

ratios of saquinavir and internal standard were plotted against the added concentrations. Linear regression without weighting was used to determine the slope and intercept.

2.6. Validation tests

The variation of retention times of saquinavir and of Ro 31-8533 versus pH was studied by replacing sulfuric acid in the mobile phase by phosphate buffer 0.01 *M*. In these experiments the composition of acetonitrile (24.5%) and the concentration of TBA (0.01 *M*) of the standard mobile phase were unchanged, whereas the pH of phosphate buffer was adjusted to 3, 4, 5, 6 by titration with 0.01 *M* NaOH. At each pH value, saquinavir (100 ng) and Ro 31-8533 (250 ng) were injected onto the column and the retention times were recorded.

The effect of the ion-paring agent TBA on the retention time of drugs was then investigated by first measuring the retention times of saquinavir, Ro 31-8533 using the standard mobile phase containing a higher concentration of TBA (0.06 *M*). The concentration of TBA was then diluted progressively to 0.007 *M* by adding a mixture of 5 m*M* H_2SO_4 and acetonitrile (75.5:24.5, v/v). At each concentration of TBA, the system was equilibrated by pumping at least 150 ml of the mobile phase to be tested through the column. The variation of the retention times was then recorded in the same manner as described for the pH.

Recovery, drug stability, precision, accuracy and limit of detection of the assay were determined using the standard conditions: (i) 5 m*M* H_2SO_4-CH_3CN (75.5:24.5, v/v) containing 10 m*M* TBA, pH 3.5; (ii) Nucleosil 3C8 column maintained at 45°C; (iii) UV detection at 240 nm.

The recovery of saquinavir by the extraction procedure was assayed by preparing three solutions of saquinavir (200, 1000, 2000 ng/ml) in the mobile phase. Fifty microliters of these solutions (equivalent to 10, 50 and 100 ng of saquinavir) were injected directly into the chromatograph. The same volumes were diluted into 1 ml of plasma, extracted with diethyl ether and analyzed as described. The ratio of peak-heights was used for calculating the extraction recovery.

The limit of detection was determined by diluting a plasma sample containing 10 ng/ml of saquinavir with drug-free plasma. Diluted samples were then extracted and analyzed. The detection limit of the assay was defined as a signal-to-base line ratio of 3:1.

The stability of saquinavir and its analogue Ro 31-8533 under diverse storage conditions was studied by extracting 400 ng of each compound from 2 ml of human plasma into 6 ml of diethyl ether. The organic phase was then divided in two parts and both tubes were evaporated to dryness. The residue in one tube was dissolved immediatly in 100 μl of mobile phase and 20 μl of the aliquot were injected twice into the chromatograph. The rest of the aliquot was stored at 22°C and re-analyzed after 16 h. The residue of the second tube was stored at 4°C and 24 h later, it was dissolved in 100 μl mobile phase and analyzed. The peak-height ratios obtained from the three occasions were calculated and compared.

Precision and accuracy of the assay were investigated in human plasma by measuring three standard samples containing different amounts of saquinavir five times on the same day and on five different days.

3. Results

The chemical structure of the saquinavir molecule is shown in Fig. 1. The UV spectrum of the drug is characterized by an absorption band at 240 nm (Fig. 2) allowing detection at this wavelength. During the development of the assay, we observed that in some human volunteers the saquinavir signal was interfered with that by endogenous substance(s). The clean base-line could be restored when TBA was added to the mobile phase. Addition of TBA to the mobile phase increased the retention time of com-

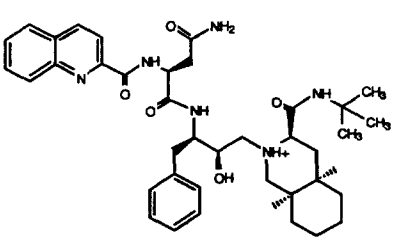

Fig. 1. Chemical structure of saquinavir.

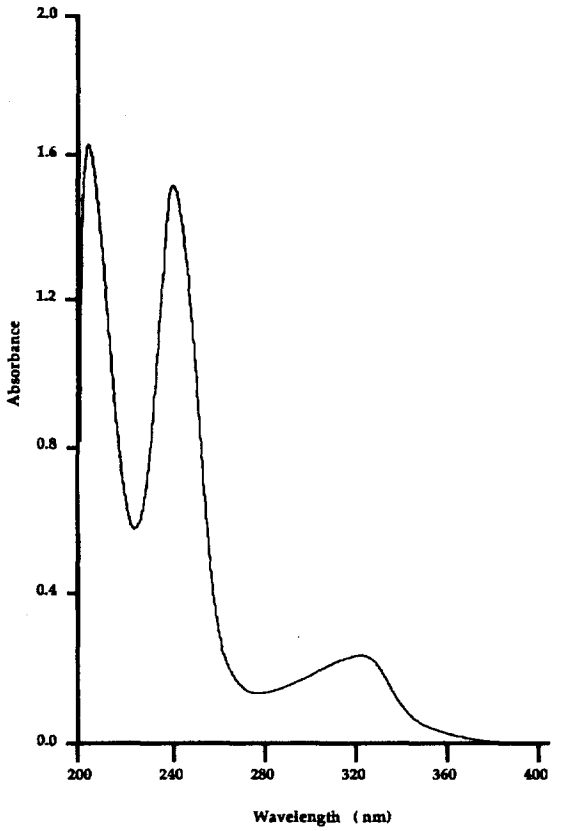

Fig. 2. UV spectrum of saquinavir mesylate $4 \cdot 10^{-5}$ *M* dissolved in HCl 0.01 *M*.

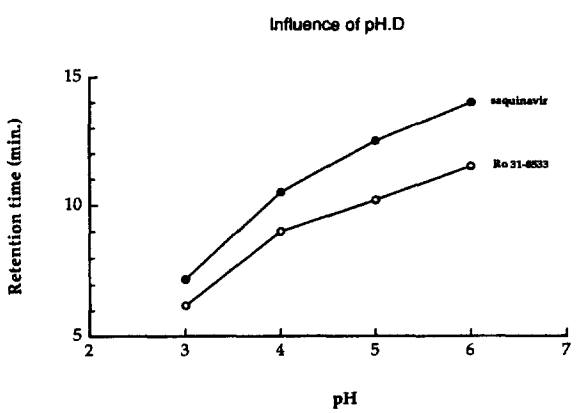

Fig. 3. Effect of pH on the retention time of saquinavir. The mobile phase was composed of potassium phosphate buffer 0.01 *M* at a pH range between 3 and 6 and acetonitrile (75.5:24.5, v/v). The concentration of the ion-pairing agent TBA was constant at 0.01 *M*.

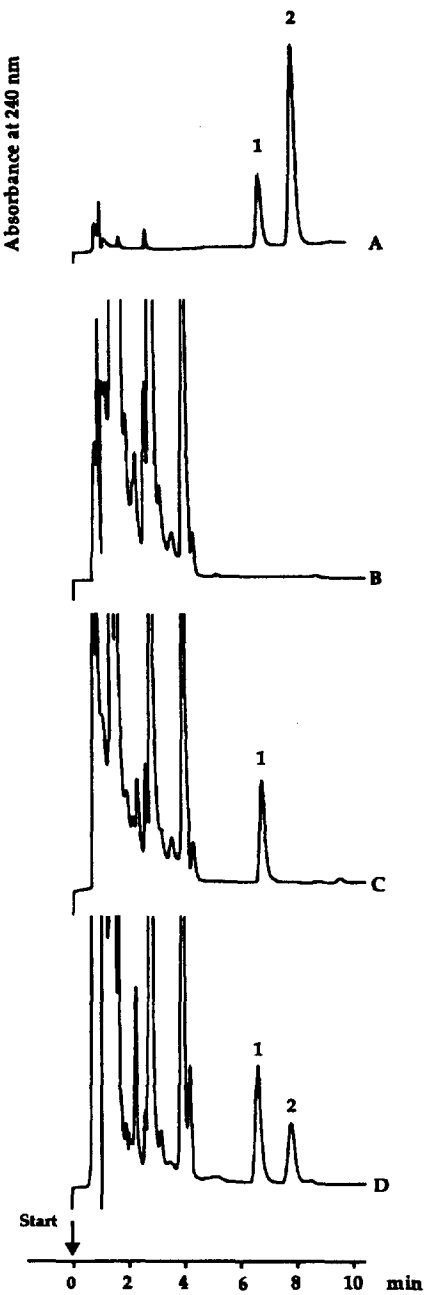

Fig. 4. HPLC chromatograms of standard substances (A); extract of 1 ml of blank human plasma: without internal standard Ro 31-8533 or saquinavir (B); spiked with 30 ng of internal standard Ro 31-8533 (C); and extract of 1 ml of plasma obtained from a volunteer receiving 600 mg of saquinavir mesylate orally. The saquinavir concentration was 20 ng/ml (D). Peak identification: (1) internal standard Ro 31-8533; (2) saquinavir. Retention times were 6.4 and 7.7 min, respectively.

pound(s) which interfered with the measured drugs, whereas those of saquinavir and Ro 31-8533 only slightly decreased with increasing concentration of TBA. As shown in Fig. 3, on a reversed-phase HPLC column, the retention times of saquinavir and its stereoisomer Ro 31-8533 increased with rising pH of the mobile phase. Under our standard conditions (see Section 2.4), saquinavir and Ro 31-5133 were separated from endogenous compounds with retention times of 7.7 and 6.4 min, respectively (Fig. 4).

To quantify saquinavir in the low range (<100 ng/ml), it is necessary to wash human plasma sample with *n*-hexane. This procedure eliminates interfering endogenous compound(s) and helps to provide a stable base-line. The drug can then be isolated by liquid–liquid extraction. Several organic solvents commonly used in the analytical laboratory such as toluene, benzene, ethylacetate and diethyl ether were used to extract saquinavir from human plasma. All of them exhibited a comparable performance with extraction recoveries >80%. Diethyl ether was selected because it gives the cleanest base-line and allows a short sample preparation time. The extraction recovery of saquinavir and its stereomer Ro 31-8533 (range 10–100 ng/ml) from human plasma into diethyl ether at basic pH was quantitative (98±5%, $n=3$) and independent of the drug concentration. This performance is comparable to that of solid-phase extraction (SPE), which has been reported previously [5]. The standard curve of saquinavir in plasma was linear over the range 2.5–100 ng/ml. The correlation coefficients were >0.99 and the intercept with the *y*-axis was not different from zero. To obtain linear calibration graphs, it is necessary, however, to prepare a saquinavir stock solution in plasma and the standard samples by dilution of this solution with drug-free plasma. If the drug is first dissolved in methanol, diluted in water and then added to plasma [final composition: water–methanol (98:2, v/v)], the calibration graph will have the form of a curve (data not shown).

Precision and accuracy of the assay were determined by analyzing plasma samples ($n=5$) containing saquinavir in the concentration range of 10–110 ng/ml on the same day (within-day) and on different days (between-day). As summarized in Table 1, the relative standard deviation (R.S.D.) for saquinavir for both within- and between-day precision were <5%. Similary, the accuracy expressed as the relative error (R.E.) was <±5% of the real value.

Saquinavir and its isomer Ro 31-8533 are stable in the methanolic and aqueous solution at 22°C for one week. In plasma, they are stable for at least three months at −20°C. We observed that saquinavir and Ro 31-8533 extracted from plasma were stable for 16 h in mobile phase at 22°C and for at least one day when the residue is stored dry at 4°C. The HPLC system was stable for at least 150 injections and routinely the precolumn was changed when the pressure raised above 250 bar.

When 1 ml of plasma was used, the limit of detection for saquinavir (defined by a signal-to-noise ratio of 3) was 1 ng/ml. In comparison, using 0.25 ml plasma, a published HPLC–MS–MS assay had a detection limit of 0.4 ng/ml [5].

Table 1
Precision (R.S.D.) and accuracy (R.E.) of saquinavir measurement in human plasma

	Added (ng/ml)	Found (ng/ml)	R.S.D. (%)	R.E. (%)
Within-day ($n=5$)	10	9.55	2.83	−4.50
	20	19.9	0.70	−0.25
	110	108.5	1.70	−1.36
Between-day ($n=5$)	10	10.2	4.41	+2.0
	20	19.7	4.07	−1.72
	80	80.3	3.24	+0.40

R.S.D.: relative standard error=(standard deviation×100)/found.
R.E.: relative error=(found−added)×100/added.

4. Discussion

This is the first HPLC assay using UV detection for the determination of saquinavir in human plasma samples. An HPLC method was also used in Roche's laboratories [6], however no details were published. The available pharmacokinetic data of saquinavir in man have been derived from drug concentrations measured by a highly selective and sensitive HPLC–MS–MS assay [5]. However, the equipment for this technique is expensive and not available for every laboratory. Therefore, we developed a sensitive HPLC assay with UV detection for saquinavir. We used a Nucleosil 3C8 HPLC column with an I.D. of 3 mm for the separation of saquinavir from its stereoisomer Ro 31-8533. In comparison with Nucleosil 5C18, packed in a conventional 125×4.6-mm I.D. column, the 3-mm I.D. column packed with Nucleosil 3C8 revealed a comparable selectivity but with an almost doubled sensitivity. However, to minimize the back pressure, it was necessary to thermostatically control the analytical column at 45°C. Concerning the mobile phase, diluted sulfuric acid was preferred to acetic acid or phosphate buffer, because sulfuric acid improved the resolution of the signals.

Since saquinavir has a low bioavailability in humans, the analytical assay must have a good performance in the low concentration range (<100 ng/ml) in plasma. In order to measure saquinavir selectively and precisely in human plasma our strategy was: (i) to remove lipophilic endogenous compounds in human plasma by *n*-hexane extraction; (ii) to isolate saquinavir from plasma by liquid–liquid extraction; (iii) to concentrate traces of drug on a 3-μm reversed-phase column; and (iv) to separate saquinavir and its related compounds from endogenous substances by ion-paired chromatography. Both extraction with hexane and use of TBA as ion-pairing reagent were necessary to obtain a clean base-line, which is important to be able to increase the sensitivity of the assay. In the presence of TBA, the retention time of the interfering compound(s) with saquinavir increased, suggesting that these endogenous substances are negatively charged. Because the ion-pair:free analyte equilibrium is very sensitive to changes in temperature and solvent composition, the analytical column should be thermostated and only a small volume (≤50 μl) of mobile phase should be used for injection.

Our studies demonstrated that saquinavir can be isolated from human plasma by liquid–liquid extraction and that diethyl ether is an appropriate solvent for the extraction. Other organic solvents, e.g. ethylacetate, toluene, benzene, may also be used for extraction with similar results. Using a solid-phase extraction (SPE) technique, Knebel et al. [5] have reported that the extraction recovery of saquinavir from human plasma was >95%. We have compared our diethyl ether extraction with SPE and found that the performance of both techniques is comparable but diethyl ether extraction, with preceding *n*-hexane extraction, is more economic and provides a more stable base-line.

It was interesting to observe that the calibration graph for saquinavir was linear in the range of 2.5–100 ng/ml only, when the methanol stock solution of the drug was diluted to the desired concentrations with drug-free plasma. If the methanol stock solution was diluted first with water and then added to plasma to reach the final concentrations, the calibration graph was not linear. One possible explanation for this finding is that saquinavir may adsorb nonspecifically to the glassware. Such loss would be drug concentration independent and would therefore explain the non-linear form of the calibration graph. For the calibration by LC–MS–MS, Knebel et al. [5] had to prepare saquinavir in water–methanol (1:1, v/v) before adding it to human plasma.

With the described method, saquinavir can be analyzed in every laboratory equiped with a conventional HPLC. One run takes less than 10 min and the assay has a stable base-line, which allows to run it with automatic injection. Routinely, one technician extracted up to 50 samples per working day with unattended overnight analysis.

The present method was used successfully for the determination of saquinavir in 720 plasma samples of eight human volunteers receiving saquinavir. Fig. 5 illustrates the plasma concentrations of a starved subject in two different occasions: after oral administration of three hard gelatine capsules containing 200 mg each of saquinavir mesylate (Fig. 5A) and after i.v. administration of 12 mg of saquinavir mesylate (Fig. 5B). After oral administration, the maximum

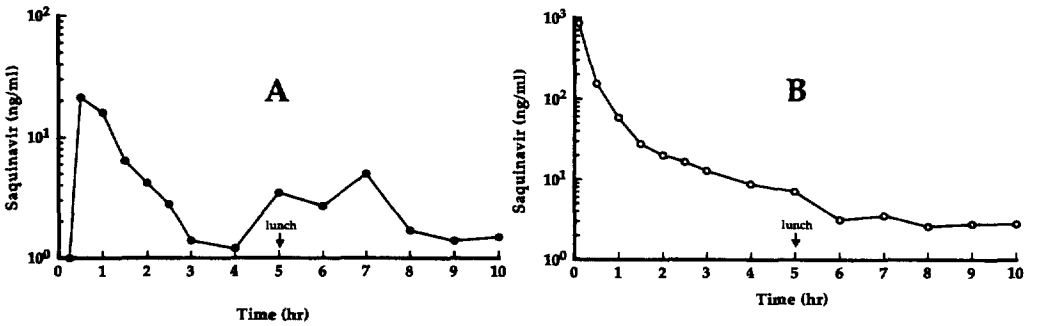

Fig. 5. Plasma concentration–time profile of saquinavir after administration of a single dose of 600 mg orally (A) or 12 mg intraveneously (B) of saquinavir mesylate to a human volunteer.

plasma concentration of saquinavir was approximately 20 ng/ml and a second plasma concentration peak was observed after 5 h, after lunch, suggesting enterohepatic circulation and/or an increase in saquinavir absorption when ingested together with food. After i.v. administration, a rapid distribution phase was followed by a slower phase of elimination and no post-prandial peak could be observed.

In conclusion, we have developed a technically simple, inexpensive, and reliable method for the determination of saquinavir in plasma. The assay can be used for pharmacokinetic studies in humans and should therefore facilitate clinical research on saquinavir.

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