ELSEVIER

Journal of Chromatography B, 714 (1998) 384–389

JOURNAL OF CHROMATOGRAPHY B

Short communication

Simultaneous determination of ritonavir and saquinavir, two human immunodeficiency virus protease inhibitors, in human serum by high-performance liquid chromatography

S. Frappier[a], D. Breilh[a,*], E. Diarte[a], B. Ba[a], D. Ducint[a], J.L. Pellegrin[b], M.C. Saux[a]

[a]*Clinical Pharmacokinetic Department, Victor Segalen University Bordeaux France and Pharmacy Haut-leveque Hospital, Magellan Avenue, 33604 Pessac, France*

[b]*Infectious Disease Department Haut-leveque Hospital, Magellan Avenue, 33604 Pessac, France*

Received 11 November 1997; received in revised form 1 April 1998; accepted 28 April 1998

Abstract

The aim of this study was to describe an high-performance liquid chromatographic assay for the simultaneous determination of two HIV protease inhibitors, saquinavir and ritonavir, in human serum. The method involved extraction of ritonavir and saquinavir from serum with the aid of solid-phase extraction C_{18} cartridges followed by high-performance liquid chromatography with a C_8 column and ultraviolet detection set at a wavelength of 240 nm. The assay was linear and has been validated over the concentrations range of 0.5–32 μg/ml for ritonavir and 0.075–4.8 μg/ml for saquinavir, from 600 μl serum extracted. In future, the assay will be used to support human population pharmacokinetic studies, and therapeutic drug monitoring for ritonavir and saquinavir.

Keywords: Saquinavir; Ritonavir

1. Introduction

Considerable effort has been expended in recent years in the development of inhibitors of the virally-encoded protease of HIV. If this enzyme is nonfunctional, immature noninfectious viruses are produced. Clinical studies showed that the protease inhibitors were very potent anti-HIV drugs with the four compounds licensed for use: ritonavir, saquinavir, indinavir and nelfinavir; all are reported to produce marked reductions in plasma viral load, particularly when used in combination with nucleoside analogue reverse transcriptase inhibitors [1,2]. Combinations of antiretroviral agents are being used to manage patients with HIV disease by attempting to improve the extent and duration of antiretroviral activity and, hence, clinical benefit, as well as limiting the development of viral resistance and drug intolerance. These combinations were chosen on the basis of the in vitro and in vivo data for their additivity or, ideally, synergy along with consideration of issues of resistance [3]. Further progress followed recent studies with three or two reverse transcriptase (RT) and a protease inhibitor producing greater antiviral activity with prolonged reductions of 2.0–3.0 log in plasma HIV RNA [4,5]. Also, in vitro works demonstrated that isolates from patients treated with ritonavir showed decreased sensitivity to ritonavir

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00220-5

but remained susceptible to saquinavir suggesting that combination of these two protease inhibitors was more effective than monotherapy [6] but the combination did raise important pharmacokinetic issues relevant to drugs interactions. The development of a high-performance liquid chromatographic (HPLC) method for the simultaneous determination of the combination ritonavir–saquinavir will be use for future studies in therapeutic drug monitoring.

2. Experimental

2.1. Chemicals

Ritonavir (ABT538) was obtained from Abbott (Rungis, France), saquinavir mesylate (lot Ro31-8959) and diazepam, the internal standard from Roche (Neuilly sur seine, France). Acetonitrile (Chromar quality) was from Touzart and Matignon (Courtaboeuf, France). Potassium phosphate monobasic, ammonium acetate, potassium hydroxide (38%) and methanol were from Prolabo (Fontenay-sous-bois, France). Drug free serum was purchased from Aquitaine transfusion establishment (Bordeaux, France).

2.2. Instrumentation

The Kontron model 400 HPLC system (Milan, Italy) consisted of a model 420 pump, a model 465 autosampler, and a model 430 UV–Vis detector connected to a model D450 integrator.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–5 m*M* potassium phosphate monobasic buffer, pH 8 (55:45, v/v). Phosphate buffer was made by dissolving 0.68 g of potassium phosphate in 1 l of water. The pH was adjusted to 8 with potassium hydroxide. The mobile phase was filtered though a Millipore (Saint Quentin en Yveline, France) filter HPLV 0.45 μm. The elution conditions were isocratic, and the mobile phase flow-rate was set at 1 ml/min. The analytical column was a Kromasil C_8 (5 μm, 150×4.6 mm) from Touzart and Matignon (Courtaboeuf, France). The sample injection volume was 25 μl. UV absorbance at a wavelength of 240 nm was used for detection. The analysis time was 11 min; the range of the detector was set at 0.05 a.u.f.s. for the first 6 min of the analysis before being changed to 0.02 a.u.f.s.

2.4. Preparation of standards

Stock solutions of ritonavir and saquinavir were prepared by dissolving the appropriate amount of ritonavir and saquinavir mesylate, accurately weighed, in methanol to yield a final drug concentration of 6400 μg/ml ritonavir and 960 μg/ml saquinavir. Then stock solutions were mixed (50:50, v/v) to obtain a combined working standard solution at 3200 μg/ml ritonavir and 480 μg/ml saquinavir. Working standards of 3200, 2400, 1600, 800, 400, 200, 100, 50 μg/ml ritonavir and 480, 360, 240, 120, 60, 30, 15, 7.5 μg/ml saquinavir were prepared by dilution of the 3200/480 μg/ml combined standard solution. Analysis standards were prepared by 100-fold dilution in drug-free serum. The resulting standards ranged in concentration from 0.075 to 4.8 μg/ml for saquinavir and from 0.5 to 32 μg/ml for ritonavir. These ranges were based on human ritonavir and saquinavir concentrations found in previous pharmacokinetic studies [7–11].

2.5. Sample pretreatment

Blood samples were collected in tubes without anticoagulant and centrifuged (10 min, 1850 *g*, 20°C) as soon as possible. Serum was decanted and heated for 30 min at 56°C to inactivate HIV virus before storing at −80°C.

2.6. Sample extraction procedure

An automatic sample processor was used for the extraction (ASPEC XL-Gilson, Villier le bel, France) with 1 ml C_{18} 100 mg cartridges (International Sorbent Technology supplied by Touzart and Matignon). Conditioning of the C_{18} cartridges with 1 ml each of methanol and methanol–water (5:95, v/v) was followed by loading 1 ml of a mixture prepared by diluting 600 μl of serum (calibration standard or patient sample) with 600 μl of 180 m*M* ammonium acetate buffer, pH 6.8. The samples are diluted with

buffer to prevent the precipitation of serum proteins on the IST cartridges. The loading flow-rate was 0.5 ml/min. Then, 1 ml of the internal standard solution (0.25 μg/ml in methanol–water, 30:70, v/v) was added. The cartridges were washed with 1 ml of water and the compounds were eluted twice with 250 μl of methanol. The solvent was evaporated at 50°C under a stream of nitrogen. The residue was reconstituted in 100 μl methanol–water (80:20, v/v) and 25 μl of the sample were injected into the chromatographic system.

2.7. Calibration and calculation procedures

Daily standard curves were constructed using the ratios of the observed peaks area of ritonavir and saquinavir and the internal standard. The unknown concentrations were computed from the unweighted linear regression equation of the peak-area ratio against concentration for the calibration curve.

2.8. Accuracy, precision and recovery

The intra-day accuracy and precision of the method were estimated from the back-calculated standard concentrations. The overall mean precision was defined by the coefficient of variation (C.V.) with relative errors from six duplicate standards analyzed on the same day. Inter-day variability was estimated from the triplicate analysis of three samples on six separate days. Recovery of ritonavir, saquinavir and diazepam after the solid-phase extraction procedure was determined by comparing observed ritonavir, saquinavir and diazepam concentrations in extracted serum to those of nonprocessed standard solutions.

2.9. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank serum samples. The following substrates were investigated for interference with the analytical method (including the sample pretreatment): amikacin, amphothericin B, azithromycin, captopril, cidofovir, clarithromycin, didanosine, erythomycin, fluconazole, foscarnet, fluoxetine, furosemide, ganciclovir, itrconazole, lamivudine, nicardipine, nifedipine, paroxetine, rifabutine, rifampicine, stavudine, vacomycine, zalcitabine and zidovudine.

2.10. Limit of detection and limit of quantitation

The limit of detection (LOD) in serum was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation is the minimum injected amount that gives precise measurements; chromatography, for example, typically requires peaks heights 10–20 times higher than baseline noise. If the required precision of the method at the limit of quantitation (LOQ) has been specified, the Eurachem [12] approach can be used. A number of samples with decreasing amounts of the analytes are injected six times. The calculated R.S.D.% of the precision is plotted against the analyte amount. The amount that corresponds to the previously defined required precision (15%) is equal to the limit of quantitation.

3. Results

3.1. Chromatographic characteristics

Fig. 1 shows chromatograms, respectively, of an extracted blank serum sample, an extracted serum containing 4 μg/ml ritonavir, an extracted serum containing 0.6 μg/ml saquinavir, an extracted serum standard containing 4 μg/ml ritonavir with 0.6 μg/ml saquinavir and a subject's 2-h postdose serum sample after taking 400 mg each of both ritonavir and saquinavir. The calculated capacity factors (k') for ritonavir and saquinavir were 3.58 and 5.10, respectively. Retention times were 4.93±0.01 min for ritonavir and 6.78±0.02 min for saquinavir (n=6). Peak symmetry was 1.08±0.02 for ritonavir and 1.04±0.03 for saquinavir, respectively.

3.2. Calibration curve

The analysis of ritonavir and saquinavir in serum exhibited excellent linearity (r^2=0.9992 and r^2=0.9998 respectively, for ritonavir and saquinavir) over the 0.5–32 μg/ml concentration range for ritonavir and the 0.075–4.8 μg/ml concentration range for saquinavir. Regression intercepts for the

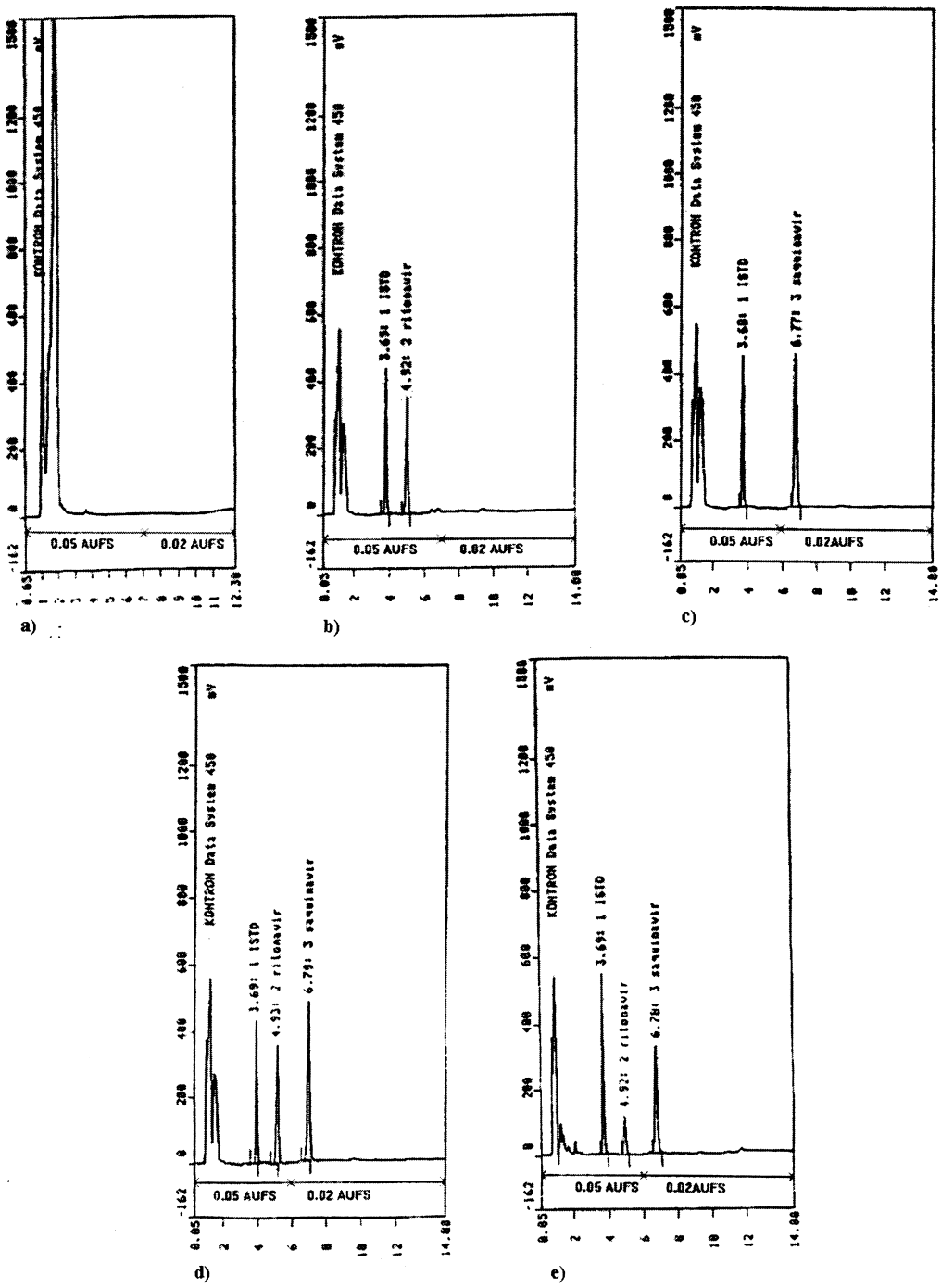

Fig. 1. Chromatograms of (a) an extracted blank serum, (b) an extracted serum containing 0.4 μg/ml ritonavir, (c) an extracted serum containing 0.6 μg/ml saquinavir, (d) an extracted serum containing 0.4 μg/ml ritonavir with 0.6 μg/ml saquinavir, and (e) a subject's 2-h postdose serum after taking 400 mg each of titonavir and saquinavir.

Table 1
Accuracy and precision of the determination of ritonavir in serum samples

Theory (μg/ml)	Found (μg/ml)	S.D.	Accuracy (%)	C.V. (%)	Relative error (%)	*n*
(a) *Intra-day*						
28	28.51	0.263	101.8	0.92	1.82	6
14	14.23	0.535	101.6	3.72	1.64	6
0.8	0.872	0.093	109	10.7	9	6
(b) *Inter-day*						
28	27.61	0.363	98.6	1.31	−1.4	18
14	14.06	0.429	100.4	3.05	0.4	18
0.8	0.725	0.089	90.6	12.3	−9.38	18

calibration curves were generally very small and were not statistically significant compared to zero. These daily calibration curves were used for calibration and calculation purposes. The results indicated that the assay had acceptable precision (<10% C.V.) for saquinavir and (<15% C.V.) for ritonavir) and accuracy (relative error <10% for saquinavir and ritonavir) to a lower LOQ of 0.05 μg/ml for saquinavir and 0.8 μg/ml for ritonavir with a 600-μl sample volume.

3.3. Accuracy, precision and recovery

The intra-day accuracy and precision of the method were estimated from the back-calculated standard concentrations. The overall mean precision, as defined by the coefficient of variation (C.V.), ranged from 0.95 to 3.2% for saquinavir and from 0.92 to 10.7% for ritonavir from six series standards analyzed on the same day (Table 1a and b). Inter-day variability, as estimated from the triplicate analysis of three samples on six separate days (Table 2a and b), was low, with C.V.s ranging from 2.39 to 8.03% for saquinavir and from 1.31 to 12.3% for ritonavir, and with relative errors ranging from −6.67 to −1.4% over the concentration range 0.120–4.200 μg/ml for saquinavir and from −9.38% to 0.4% over the concentration range 0.8–28 μg/ml for ritonavir. These results indicated that this assay was reliable and reproducible.

The extraction recoveries of ritonavir and saquinavir from quality control samples are 97 and 96% respectively. The recovery of the internal standard, as evaluated at a concentration of 0.25 μg/ml, was 96%.

3.4. Specificity and selectivity

Blank serum showed no interfering endogenous substances in the analysis of ritonavir and saquinavir. Potentially coadministered drugs tested had retention times that were different from saquinavir and ritonavir or were not detected with the described bioanalytical method.

Table 2
Accuracy and precision of the determination of saquinavir in serum samples

Theory (μg/ml)	Found (μg/ml)	S.D.	Accuracy (%)	C.V. (%)	Relative error (%)	*n*
(a) *Intra-day*						
4.200	4.197	0.040	99.93	0.95	−0.07	6
2.100	2.156	0.038	102.7	1.76	2.7	6
0.120	0.126	0.004	105	3.2	4.2	6
(b) *Inter-day*						
4.200	4.315	0.103	102.7	2.39	−1.4	18
2.100	2.057	0.078	97.9	3.79	−2.09	18
0.120	0.112	0.009	93.3	8.03	−6.67	18

3.5. Limit of detection and limit of quantitation

The LODs in serum were 0.2 μg/ml for ritonavir and 0.02 μg/ml for saquinavir. At this concentration the signal-to-noise ratio was 3. At 0.8 μg/ml ritonavir and 0.05 μg/ml saquinavir the percent deviations from the respective nominal concentrations and the R.S.D. were both less than 16%. Thus, 0.8 μg/ml ritonavir and 0.05 μg/ml saquinavir were defined to be the LLQ.

4. Discussion

A highly specific, sensitive and rapid method has been developed for the quantitation of ritonavir and saquinavir in human serum samples. The applicability of the assay for pharmacokinetic research in HIV-1 infected patients was possible. Serum pharmacokinetics of ritonavir, saquinavir and other protease inhibitors showed large inter-individual variation caused by low or variable oral bioavaibility of the currently formulations of ritonavir and saquinavir and by extensive cytochrome P450 metabolism of this drug. Indications of a pharmacokinetic–pharmacodynamic relationships have been reported, suggesting enhanced antiviral efficacy with increased exposure to saquinavir. Ritonavir appeared to be well tolerated but data from clinical trials showed that adverse events occurred in the period of drug initiation. Resistance or reduced sensitivity to the leading protease inhibitors has been reported and appeared to be associated with loss of therapeutic effect. Thus, monitoring saquinavir and ritonavir pharmacokinetics may be imperative to ensure optimal drug efficacy, to prevent the risk of drug resistance in individual patient and to control the tolerability profile during the initiation of treatment. In conclusion, a validated assay for the quantitation determination of ritonavir, saquinavir in human serum has been described. The currently described HPLC assay can readily be used in a hospital laboratory for the monitoring of ritonavir and saquinavir combination concentrations.

References

[1] G. Moyle, B. Gazzard, Drugs 51 (1996) 701.
[2] R. Gulick, J. Mellors, D. Havlir, XI International Congress on AIDS, Vancouver, July 1996, abstract Th.B 931.
[3] E. De Clercq, Clin. Microb. Ref. 8 (1995) 200.
[4] R.T. D'Aquila, M.D. Hughes, V.A. Johnson, Ann Intern Med. 124 (1996) 1019.
[5] R. Gulick, J. Mellors, D. Havlir, Third Conference on Retroviruses and Opportunistic Infections, Washington, January–February 1996, abstract LB7.
[6] M. Markowitz, H. Mo, D.R. Kempi, J. Virol. 69 (1995) 701.
[7] R.M.W. Hoetelmans, M. van Essenberg, P.L. Meehorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B 698 (1997) 235.
[8] N.J. Knebel, S.R. Sharp, M.J. Madigan, J. Mass Spectrom. 30 (1995) 1149.
[9] E.J. Wu, J.M. Wilkinson, D.G. Naret, J. Chromatogr. B 695 (1997) 373.
[10] E. Woolf, T. Au, H. Haddix, J. Chromatogr. A 692 (1995) 45.
[11] H.R. Ha, F. Follath, Y. Bloemhard, S. Krahenbuhl, J. Chromatogr. B 694 (1997) 427.
[12] L. Huber, LC·GC Int., February (1998) 96–105.