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High-performance liquid chromatographic determination of ritonavir in human plasma, cerebrospinal fluid and saliva

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Abstract

A simple, ion-pair high-performance liquid chromatographic method has been developed and validated for the quantitative determination of the HIV protease inhibitor ritonavir in human plasma, cerebrospinal fluid and saliva. Sample pretreatment consisted of precipitation of proteins with acetonitrile prior to high-performance liquid chromatography with ultraviolet detection at 239 nm. The method has been validated over the range of 50 ng/ml to 50 μg/ml with use of 100-μl volumes of sample. The currently described assay has been used successfully for the analysis of ritonavir in plasma, cerebrospinal fluid and saliva in HIV-1 infected patients. © 1998 Elsevier Science B.V.

Keywords: Ritonavir

1. Introduction

Ritonavir (ABT-538, Norvir, Fig. 1) is an HIV protease inhibitor (a new class of antiretroviral drug), and is a potent in vitro and in vivo inhibitor of the human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS) [1–3]. Ritonavir was approved by the US Food and Drug Administration (FDA) for single use, or in combination with approved antiretroviral nucleoside analogues in patients with advanced HIV infection; approval was based on data that demonstrated delays in disease progression and death. Ritonavir was also approved by the FDA, under its accelerated approval regulations, for patients with early HIV infection based on an observed beneficial effect of ritonavir on surrogate parameters. In the European Union ritonavir was the first HIV protease inhibitor to be licensed in August 1996 for use in combination with antiretroviral nucleoside analogue(s) in HIV infected adult patients with advanced or progressive immunodeficiency [4]. The pharmacokinetic profile of ritonavir in HIV infected

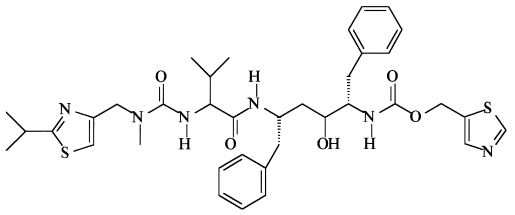

Fig. 1. Molecular structure of ritonavir.

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patients has recently been described, although not in the recommended clinical dosage of 600 mg bid [5]. Ritonavir is a very potent inhibitor of cytochrome P450 (CYP) isozymes, in particular CYP3A4 and CYP2D6 [4]. Furthermore, ritonavir is capable of inducing the glucuronidation of several drugs. Ritonavir also induces its own metabolism at the start of treatment [4]. Thus, the drug-interaction potential of this novel compound is impressive and a ready to use bioanalytical method to study potential drug interactions with ritonavir in HIV infected patients is warranted. Furthermore, indications of pharmacokinetic–pharmacodynamic relationships have been reported, suggesting that the monitoring of ritonavir plasma concentrations might prove to be of value [2,6].

Recently, a bioanalytical method for the determination of ritonavir in human samples was briefly described [5]. We report the development and full validation of a simple, ion-pair high-performance liquid chromatographic (HPLC) assay with ultraviolet (UV) detection for the quantitative determination of ritonavir in human plasma, cerebrospinal fluid and saliva. This assay has been 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 ritonavir solutions in 50% (v/v) methanol were recorded with a Model 918 UV–Vis spectrophotometer (GBC Scientific Equipment, Dandenons, Australia).

2.2. Chemicals

Ritonavir (Lot 324085AX) was a kind gift of Abbott Laboratories. Acetonitrile (HPLC supra-gradient) and methanol (supra-gradient) were purchased from Biosolve (Valkenswaard, Netherlands). Hydrochloric acid 37% analytical-reagent grade, sodium acetate, and hexane-1-sulfonic acid (sodium salt) both analytical-reagent grade 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, Netherlands). Blank cerebrospinal fluid was obtained from patients who underwent lumbar puncture for methotrexate monitoring after intrathecal administration of this drug. Blank saliva was obtained from volunteers.

2.3. Preparation of standards

Stock solutions of ritonavir were prepared by dissolving the appropriate amount of ritonavir, accurately weighed, in 50% (v/v) methanol to yield a final drug concentration 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, 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 ritonavir were diluted with 50% (v/v) methanol. To achieve ritonavir calibration concentrations of 50 to 50 000 ng/ml, appropriate quantities of the various diluted solutions were added to blank plasma, cerebrospinal fluid, or saliva in Eppendorf tubes (Merck). The solutions were vortex-mixed for 10 s. Next, to 100 μl of plasma, cerebrospinal fluid, or saliva samples a volume of 400 μl of acetonitrile was added to precipitate proteins. After vortex-mixing for 30 s the samples were centrifuged for 3 min at 10 500 *g*. Then, 400 μl of the clear supernatant was evaporated to dryness in Eppendorf tubes under a gentle stream of nitrogen at 40°C. The residues

were redissolved in 150 μl of mobile phase, vortex-mixed 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% (44:56, v/v). Prior to use, air was removed by bleeding 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, cerebrospinal fluid and saliva samples. The following compounds were investigated for interference with the analytical method: didanosine, fluconazole, folinic acid, ganciclovir, indinavir, lamivudine, methadone, methotrexate, nelfinavir, oxazepam, pyrazinamide, ranitidine, rifampin, saquinavir, 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 ritonavir. 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 50 μg/ml.

2.8. Accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precision of the method were determined by assaying six replicate samples of plasma at three different ritonavir concentrations (499, 4988 and 19 952 ng/ml) in three analytical runs. These samples were made independent of standard curve concentrations. Accuracy was measured as the percent deviation from the nominal concentration. The within-day and between-day precision 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 $1/(\text{conc.})^2$ [7,8]. For the construction of each calibration curve 12 spiked plasma samples were analyzed in duplicate.

Recovery of ritonavir after the sample pretreatment procedure was determined by comparing observed ritonavir concentrations in extracted plasma, cerebrospinal fluid and saliva samples to those of nonprocessed standard solutions.

2.9. Stability

Blank plasma samples were spiked with an aliquot of diluted ritonavir stock solution to give initial concentrations of 5035 and 20 140 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. One additional set of samples underwent three freeze–thawing cycles. After the storage period six replicates of the samples were analyzed and compared to six replicates of control samples.

2.10. Analysis of patient samples

HIV-1 infected patients on ritonavir therapy (600 mg every 12 h) ingested the drug with a breakfast after an overnight fast. 12 heparinized blood samples were drawn during a time period of 8 h. Plasma was separated by centrifugation at 3000 *g* at 4°C for 10 min and was immediately stored at −30°C until

analysis. Nine saliva samples were also collected with a Salivette collecting device (Sarstedt, Etten-Leur, 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.

From one patient a cerebrospinal fluid sample and a concomitantly drawn plasma sample were available (2 h after ingestion of a 600 mg ritonavir dose).

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

We used a recently developed bioanalytical assay for the determination of saquinavir, a structurally similar HIV protease inhibitor, as the basis for the currently described method [9]. Considering the basic character of ritonavir, an endcapped analytical column was used to reduce the risk of tailing peaks [Zorbax SB-C_{18} column (75×4.6 mm I.D., particle size 3.5 μm)]. Ritonavir displays significant UV absorption. Thus, UV detection was performed at 239 nm since this was the wavelength of maximal absorption of ritonavir in the mobile phase (specific extinction 119).

Reversed-phase chromatography was performed with various mixtures of acetonitrile and 25 m*M* sodium acetate solution. The pH value of the aqueous phase was adjusted to 4.0 with a 4 *M* hydrochloric acid solution in distilled water. Peak shape and separation from endogenous compounds were optimised by the addition of hexane-1-sulfonic acid. A concentration of 25 m*M* proved to be satisfactory 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 saquinavir or indinavir) as an internal standard was not considered, since we were increasingly confronted with patients in whom ritonavir plus saquinavir or indinavir were coadministered. Furthermore, the assay as described gives satisfactory validation results without the use of an internal standard.

3.2. Sample pretreatment and recovery

For the analysis of saquinavir a solid-phase extraction (SPE) methodology is used [9,10]. Though laborious, the procedure of SPE in that assay is necessary since concentrations of saquinavir in plasma are in the ng/ml range. For ritonavir, however, clinically therapeutic concentrations are in the μg/ml range and we decided to investigate whether the precipitation of proteins would be sufficient as a sample pretreatment procedure. A volume of 400 μl of acetonitrile was added to 100 μl of sample to precipitate proteins. Direct injection of the clear solution onto the analytical column resulted in poor peak shape, most likely due to the presence of large amounts of acetonitrile. Therefore, 400 μl of the diluted sample was evaporated to dryness with use of a gentle stream of nitrogen at 40°C and the residue was dissolved in 150 μl of mobile phase. After centrifuging and injecting 100 μl of the clear supernatant good peak shape was obtained.

Recovery of ritonavir was determined and calculated by comparing observed ritonavir concentrations in extracted samples to those of nonprocessed standard solutions. For plasma samples the recovery ranged from 102 to 104%, for cerebrospinal fluid samples from 99 to 101%, and for saliva samples from 100 to 105% (Table 1).

3.3. Specificity and selectivity

Blank plasma, cerebrospinal fluid and saliva samples from six different individuals showed no interfering endogenous substances in the analysis of ritonavir (Fig. 2A–C). Potentially coadministered drugs or metabolites that were tested had retention times that were different from ritonavir that eluted at approximately 9 min [saquinavir (5 min), indinavir (2 min), nelfinavir (6 min) and rifampin (7 min)], or were not detected with the described bioanalytical

Table 1
Recoveries of ritonavir from spiked samples

Ritonavir concentration (ng/ml)	Recovery (mean±S.D.) (%)
Plasma[a]	
532	102±1.2
5320	102±4.2
21 280	104±2.6
Cerebrospinal fluid[b]	
468	99
4678	97
18 900	101
Saliva[b]	
468	105
4678	102
18 900	100

[a] Determined in three analytical runs.
[b] Determined in one analytical run.
Abbreviations: S.D.=standard deviation.

method. The elution of endogenous compounds with a retention time of approximately 18 min necessitated a run time of 20 min.

3.4. Limit of detection and limit of quantitation

The LOD in plasma was 20 ng/ml. At this concentration the signal-to-noise ratio was 3. In addition, the response was significantly different from blank plasma ($p<0.001$). At 50 ng/ml the percent deviation from the nominal concentration and the relative standard deviation were both less than 20%. Thus, 50 ng/ml was defined to be the LLQ. At all other concentrations up to the ULQ (50 μg/ml) the percent deviation from the nominal concentration and the relative standard deviation were less than 15%. A typical chromatogram of a patient plasma sample of 470 ng/ml is shown in Fig. 2D.

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 $1/(\text{conc.})^2$ resulted in a minimal deviation from nominal concentrations. The method proved to be accurate (average accuracy at three concentrations 96 to 100% of the real concentrations) and precise (within-day precision ranged from 0.7 to 5.7%). Some additional variation was observed as a result of performing the assay on different days (between-day precision ranged from 2.7 to 6.8%). Correlation coefficients (r^2) of calibration curves were >0.99 as determined by least squares analysis.

Calibration curves proved to be linear in the range of 50–50 000 ng/ml with use of the *F*-test for lack of fit as an indicator of linearity of the regression model.

The stability of ritonavir at various conditions is shown in Table 3. Under all conditions tested ritonavir is stable with concentrations of at least 92% of the initial concentration.

3.6. Analysis of patient samples

Indications of pharmacokinetic–pharmacodynamic relationships have been reported [2,6]. Thus, monitoring ritonavir pharmacokinetics may prove to be important in clinical practice to ensure optimal drug efficacy and to prevent the risk of drug resistance in individual patients.

The applicability of the currently described assay for pharmacokinetic research in HIV-1 infected patients was demonstrated.

The plasma concentration–time profile in a patient after ingestion of 600 mg of ritonavir as determined by the currently described bioanalytical method is shown in Fig. 3. In one patient the concentration of ritonavir in cerebrospinal fluid has been determined with the currently described method and was found to be 58 ng/ml. The concentration of a concurrently drawn plasma sample was 7.81 μg/ml. Thus, penetration of ritonavir into cerebrospinal fluid seems to be low. Ritonavir concentrations in nine saliva samples were determined in one patient after ingestion of 600 mg of ritonavir. Concentrations ranged from 53 to 108 ng/ml and the mean saliva to plasma concentration ratio was 0.8%. The observed low concentrations of ritonavir in cerebrospinal fluid and saliva may be explained by high plasma protein binding of ritonavir (99%) [2].

In conclusion, a simple, validated assay, that can readily be used in any (hospital) laboratory, for the quantitative determination of ritonavir in human

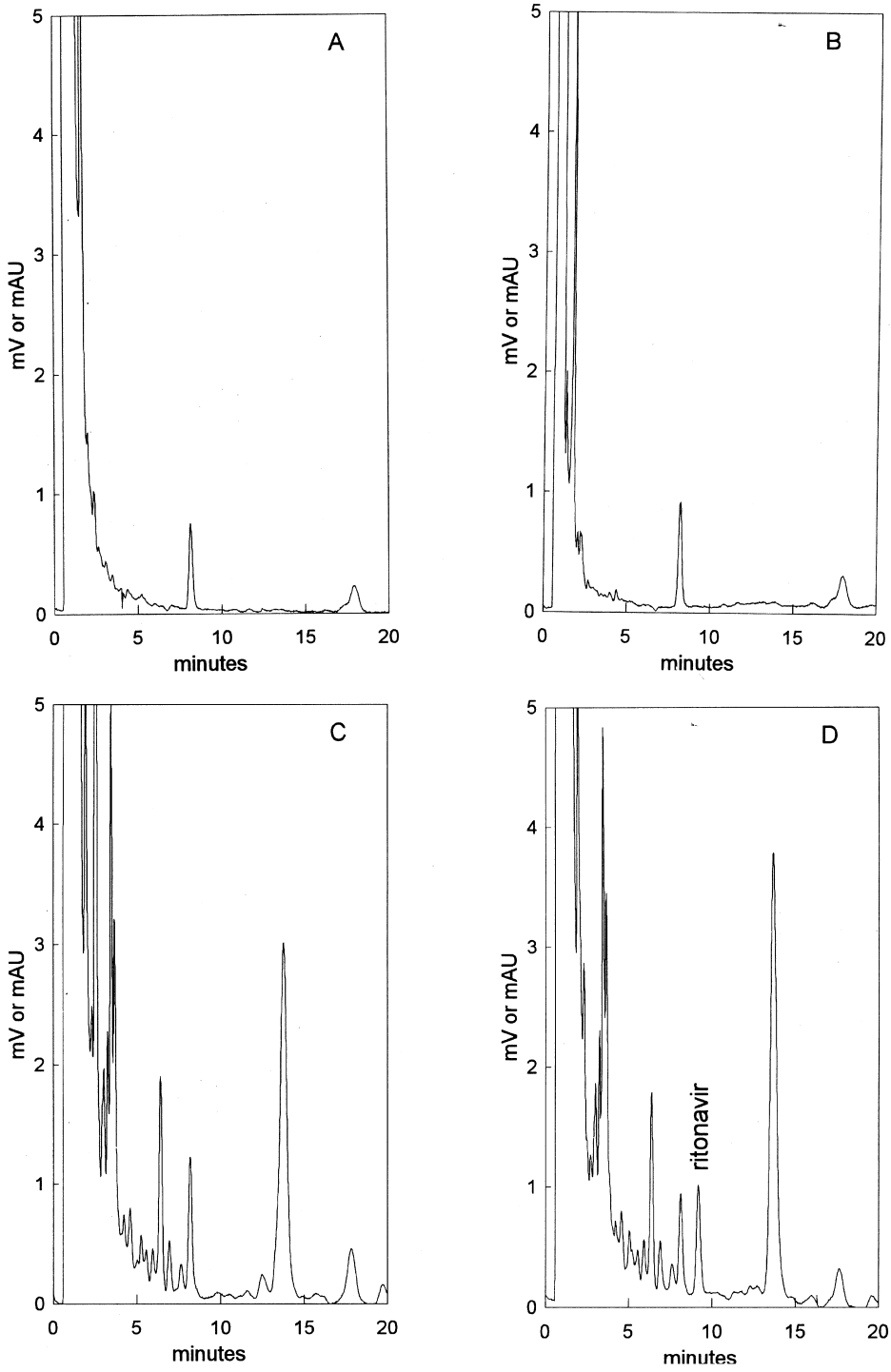

Fig. 2. Chromatograms of blank saliva (A), blank cerebrospinal fluid (B), blank plasma (C) and a patient plasma sample of 470 ng/ml ritonavir (D).

Table 2
Accuracy and precision of the analysis of ritonavir in human plasma

Concentration (ng/ml)	Accuracy (%)	90% C.I. of accuracy (%)	Precision (%)		*N*
			Between-day (N_B=3)	Within-day (N_W=6)	
499	96	86–106	6.8	5.7	18
4988	98	93–103	2.7	2.7	18
19 952	100	97–103	3.8	0.7	18

Abbreviations: C.V.=coefficient of variation, C.I.=confidence interval, N_B=number of different runs, N_W=number of replicates per run, *N*=total number of replicates.

Table 3
Stability of ritonavir in spiked human plasma samples

Storage condition	Concentration (ng/ml)	Recovery (%)	C.V. (%)	*N*
1 h at 60°C	5035	96	2.1	6
	20 140	101	2.0	6
24 h at 25°C	5035	99	0.6	6
	20 140	103	1.2	6
7 days at 4°C	5035	98	1.6	6
	20 140	96	1.2	6
30 days at −30°C	5035	92	0.8	6
	20 140	101	1.9	6
Three freeze–thaw cycles	5035	92	0.7	6
	20 140	100	1.2	6

Abbreviations: C.V.=coefficient of variation, *N*=number of replicates.

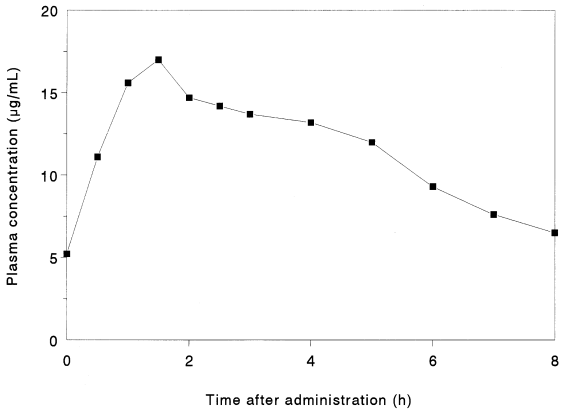

Fig. 3. Plasma concentration versus time curve of 600 mg ritonavir after oral administration with breakfast to an HIV-1 infected patient (chronic use of 600 mg ritonavir bid). A 12-h blood sample was not collected. The patient concomitantly ingested zidovudine and lamivudine.

plasma, cerebrospinal fluid and saliva is described. The assay meets the current requirements as to the validation of a bioanalytical method, covers the concentration range of interest and can be used for pharmacokinetic studies with ritonavir in HIV infected patients.

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