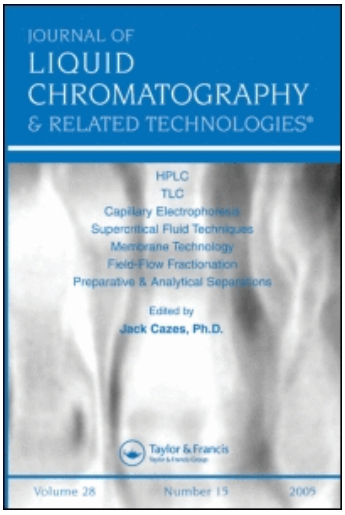

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ABSTRACT

A method is described for the simultaneous determination of protease inhibitors Indinavir, Saquinavir, and Ritonavir in human plasma using liquid chromatography with ultraviolet and electrochemical detection. Sample pre-treatment consisted of solid-phase extraction prior to ion-pair reversed phase high performance liquid chromatography with ultraviolet detection at 240 nm (Saquinavir and Ritonavir) and electrochemical detection at + 750 mV (Indinavir and Saquinavir). The method was validated with respect to specificity, linearity, limits of detection and quantifica-

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tion, precision, and accuracy. The proposed LC assay was utilized to analyze plasma samples from patients treated with HIV-protease inhibitors.

INTRODUCTION

Human immunodeficiency virus (HIV) protease inhibitors are new and potent antiretroviral drugs that have changed the treatment of infection with HIV dramatically. The HIV protease enzyme is responsible for post-translational processing of *gag* and *gag-pol* polyprotein precursors, and its inhibition by drugs such as Indinavir, Saquinavir, and Ritonavir results in the production of non infectious virions(1).

Recent advances in the treatment of HIV-1 infection involving administration of protease inhibitors have led to considerable improvements in life expectancy of infected individuals(2). Because of the increasing number of available antiretroviral drugs, the number of different combinations is rapidly growing. There is no definite data regarding superiority of one acceptably potent initial regime over another, but current recommendations favor the use of combinations of antiretroviral agents for HIV-1 therapy(3). The regimens studied to date, have included three drugs from at least two different classes of compounds having different modes of action,(4,5) but some very promising combination regimes contain two protease inhibitors(6,7). The measurement of protease inhibitor levels may have a clinical role, provided that antiviral efficiency and drug toxicity can be managed with this information(3-5,8).

Several liquid chromatographic methods with UV detection (LC-UV) or mass-spectrometry detection (LC/MS/MS), to quantify plasma concentration of protease inhibitors have been described(9-11,12-15). Methods for the simultaneous determination of PIs have been published too(8,16-22).

This work presents a relatively simple LC method for the simultaneous quantitation of protease inhibitors (indinavir, saquinavir, and ritonavir) in human plasma based on the use of a solid-phase extraction, a spectrophotometric UV detector (UV), and an electrochemical detector (ED) connected in series. The method represents an improvement in our previous study(23) and it is likely to be used with standard laboratory equipment.

EXPERIMENTAL

Chemicals and Reagents

Indinavir was supplied by Merck (Whitehouse Station, NJ, USA). Saquinavir was supplied by Glaxo Wellcome (London, UK) and Ritonavir was a

kind gift of Abbott Laboratories (Illinois, IL, USA). Acetonitrile and methanol (Lab-Scan Analytical Science, Dublin, Ireland) were HPLC grade. Hydrochloric acid 37% and anhydrous sodium acetate were purchased from Carlo Erba (Carlo Erba reagenti, Rodano, MI, Italy). 1-Hexanesulfonic acid (sodium salt) was purchased from Sigma (Sigma chemical, St. Louis, MO, USA). Water was bidistilled and all the other chemicals were of analytical reagent grade.

Standard Solutions

Stock solutions of Indinavir, Saquinavir, and Ritonavir were prepared by dissolving the appropriate amount of drug, accurately weighed, in acetonitrile: water (50:50, v\v) to yield a final concentration of 1 mg/mL. All stock solutions were stored at -20°C. Working solutions were prepared by dilution of stock solution with mobile phase.

For the preparation of standard samples, working solutions were added to blank plasma. The solutions were mixed on a vortex mixer for 10 s and used as quality control.

Sample Extraction Procedure

Prior to solid-phase extraction, Water Oasis HLB 1cc (30 mg) extraction columns were rinsed with 1.0 mL of methanol, followed by 1.0 mL of distilled water. Next, 200 µL of plasma was transferred onto the column and drawn into it. The column was then washed twice with 1.0 mL of a mixture of methanol and 0.01 M ammonia (5:5, v/v). Elution of the absorbed analyte was eluted with 1.5 mL of a mixture of methanol and 1 M acetic acid (8:2, v/v) and evaporated under vacuum at 25°C. The dried residue was reconstituted with 100 µL of mobile phase. Aliquots (20 µL) of the resulting solution were analyzed directly by LC.

Chromatographic Conditions

The HPLC system consisted of a Series 200 LC pump (Perkin Elmer, Norwalk, CT, USA), a pulse damper LP-21 (SSI, State College, PA, USA), a Rheodyne Model 7125 injection valve with a 20 µL sample loop (Rheodyne, Berkeley, CA, USA), and a Coulochem model 5100A electrochemical detector (ESA, Belford, MA, USA) equipped with an analytical cell (Model 5011). The working parameters for electrochemical detector were + 400 mV for the first electrode and + 750 mV for the second. The signal generated by the second electrode was used for the quantitation. For ED data collection and calculations a Waters Maxima 820 Chromatography workstation (Waters Corporation, Milford,

Massachusetts, USA) was used. Single-wavelength detection using a photodiode array detector model 235 (Perkin-Elmer) set at 240nm was performed. For UV data collection and calculations a Nelson model 1020 workstation (Perkin Elmer) was used.

The chromatographic analysis was performed at ambient temperature using a 3 μm Supelcosil ABZ + Plus column, 100 x 2.1 mm I.D. (Supelco Park, Bellefonte, PA, USA) and a mobile phase composed of acetonitrile plus distilled water containing 25 mM sodium acetate and 25 mM hexane-1-sulfonic acid and adjusted to pH 5.0 with hydrochloric acid 37% (40:60, v/v). The mobile phase was filtered through a 66 nylon membrane (0.45 μm) before use. The flow-rate was maintained at 0.15 mL/min. Aliquots of 20 μL were injected. The UV and ED detectors were connected in series with UV as first detector.

Analysis of Plasma Samples

Plasma concentrations of HIV-1 infected patients were determinated by the currently described method. Plasma was separated by centrifugation at 3000 x *g* for 10 min. and immediately stored at – 20°C until analysis.

Before extraction, the separated plasma was heated to 56°C for 45 minutes to inactivate HIV. The test, performed in triplicate for one low and one high concentration of each compound, ensured that there was no degradation during the inactivation of plasmas. Anti-retroviral information and drug therapy for these patients are noted in Table 1.

Table 1. Antiretroviral Therapy of HIV-1 Infected Patients

Patient	Viremia	CD4	Antiretroviral Drugs	Patient	Viremia	CD4	Antiretroviral Drugs
1	< 80	762	Indinavir 800 mg^{tid}*	11	190000	0	Saquinavir 600 mg†
2	<80	260	Saquinavir 600 mg†	12	86000	277	Indinavir 800 mg^{bid}*
3	< 80	285	Indinavir 800 mg^{bid}*	13	< 80	468	Indinavir 800 mg^{bid}*
4	4600	899	Saquinavir 600 mg^{tid}#	14	790	373	Indinavir 800 mg^{bid}
5	< 80	2185	Indinavir 800 mg^{tid}§	15	< 80	448	Saquinavir 400 mg^{tid}§
6	980	257	Saquinavir 400 mg^{tid}*	16	300	345	Ritonavir 600 mg^{bid}
7	150	895	Saquinavir 400 mg^{tid}*	17	850	378	Indinavir 800 mg^{tid}
8	< 80	143	Indinavir 800 mg^{tid}§	18	< 80	120	Indinavir 800 mg^{tid}
9	150	495	Saquinavir 600 mg^{tid}*	19	< 80	398	Indinavir 800 mg^{tid}*
10	3300	319	Indinavir 800 mg^{tid}*	20	963	421	Ritonavir 600 mg^{bid}

bid: twice a day, tid: three times per day; * in combination with lamivudine and stavudine; § in combination with lamivudine and zidovudine; † in combination with stavudine and nelfinavir; # in combination with didanosine and lamivudine.

RESULTS AND DISCUSSION

The specific method reported here for the simultaneous determination of protease inhibitors in human plasma employs HPLC with two different detection systems (UV and ED detection) connected in series. For ED, the value of + 400 mV for the first electrode was chosen because it permitted the removal of compounds with a lower oxidation potential than the analyte, which can interfere with the detection assay. The potential of + 750 mV for the second electrode was used because the response of Indinavir reached a plateau at this value.(21) For UV detection, 240 nm was chosen because Saquinavir and Ritonavir possess significant absorption at this wavelength. Figure 1 shows chromatograms of a standard mixture containing, Indinavir, Saquinavir and Ritonavir.

A suitable internal standard was not available at the time of the assay development. However, satisfactory validation results were obtained without the use of an internal standard.

Specificity

The specificity of the assay was determined by the analysis of blank plasma with possible interfering drugs (methotrexate, trimethoprim, rifampicin, lorazepam, and oxazepam). There were no interfering peaks at the retention time for protease inhibitors. Potentially coadministered drugs tested (lamivudina, stavudina and didanosina) had different retention times or were not detected by the described method.

Linearity and Limits of Detection and Quantitation

Using the conditions described above, the detection limits, quantitation limits and linearity were determined.

The limits of detection (LOD) at a signal-to-noise ratio of 3 were: 10 ng/mL for Indinavir, 15ng/mL for Saquinavir ED and 12 ng/mL for Saquinavir UV, 12 ng/mL for Ritonavir. The limits of quantitation (LOQ) were established to be 25 ng/mL for Indinavir and Saquinavir and 250 ng/mL for Ritonavir. These values were included in the calibration curve as the lowest concentration level.

The linearity was calculated by linear regression in the range from 25 to 1000 ng/mL for Indinavir and Saquinavir; from 250 to 5000 ng/mL for Ritonavir; giving a correlation coefficient > 0.999 for all protease inhibitors. The equations for the calibration curve were: Indinavir, $y = 610.49x - 6286.7$; Saquinavir (ED detection) $y = 8007.5x + 5186.4$; Saquinavir (UV detection) $y = 2324.9x - 10317$; Ritonavir, $y = 276.44x - 355.86$.

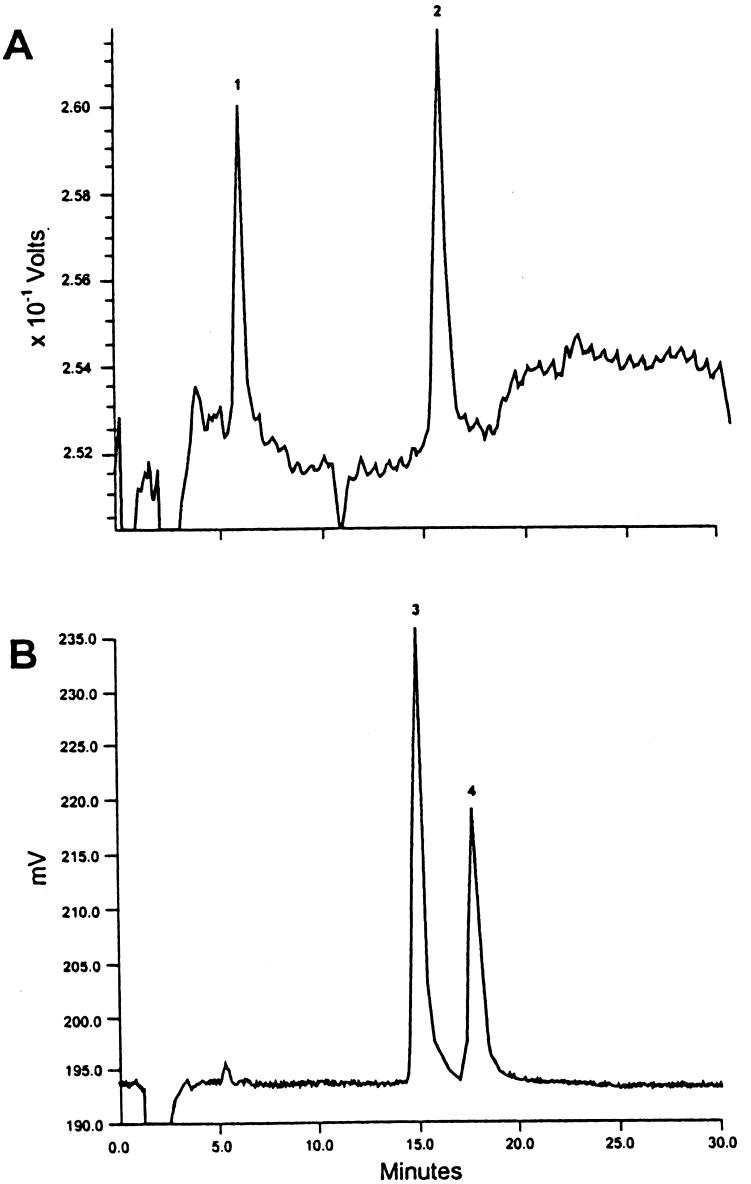

Figure 1. Chromatograms showing ED (A) and UV (B) responses of standard mixture: peak 1 Indinavir (8 ng injected on column), peak 2-3 Saquinavir (8 ng injected on column) and peak 4 Ritonavir (80 ng injected on column); column: Supelcosil ABZ + Plus column, 100 x 2.1 mm ID, 3 μm; Mobile phase: acetonitrile - water containing 25 mM sodium acetate and 25 mM 1-hexanesulfonic acid and adjusted to pH 5.0 with hydrochloric acid 37% (40:60, v/v); isocratic flow: 0.15 mL/min.

Precision and Accuracy

In Table 2, precision and accuracy are reported for human plasma. The intra-day precision calculated as the coefficient of variation (CV%) was: 1.5 to 2.5 for Indinavir, 0.9 to 5.9 for Saquinavir and 1.9 to 5.1 for Ritonavir. The inter-day precision was always below 10% for all protease inhibitors. The intra day and inter-day accuracy is defined as the agreement between the measured value and the true value (Bias%) and they were < 15% for all protease inhibitors (24).

Recovery

Recovery was determined by comparing the peak areas obtained by blank human plasma spiked with known amounts of protease inhibitors to the peak areas of identical concentrations prepared in the mobile phase without extraction. The average recoveries were 89.0% for Indinavir, 86.0% for Ritonavir, 95.0% for Saquinavir ED and 94.0% for Saquinavir UV (Table 3).

Table 2. Determination of Protease Inhibitors in Human Plasma: Precision and Accuracy

Compounds	Nominal Standard Concentration (ng/mL)	Calculated Concentration (ng/mL) Mean ± SD	Intra-Day Precision (CV%)[a]	Inter-Day Precision (CV%)[b]	Intra-Day Accuracy (Bias%)[a]	Inter-Day Accuracy (Bias%)[b]
Electrochemical detector						
Indinavir	200	179.4 ± 2.8	1.5	4.9	10.4	14.1
	300	258.5 ± 6.5	2.5	2.1	13.8	14.5
	400	368.7 ± 8.3	2.2	6.1	7.8	12.5
Saquinavir	200	190.8 ± 1.9	1.0	1.3	4.6	5.4
	300	270.3 ± 2.5	0.9	3.5	9.9	12.4
	400	392.8 ± 8.9	2.3	7.9	1.8	9.3
UV detector						
Saquinavir	200	194.0 ± 11.4	5.9	8.8	4.0	10.4
	300	288.4 ± 11.3	3.9	6.9	4.5	8.6
	400	356.7 ± 5.5	1.5	9.9	10.8	13.5
Ritonavir	1000	882.1 ± 40.0	5.1	7.3	11.8	13.4
	5000	446.3 ± 82.5	1.9	6.2	10.8	14.6
	10000	8017 ± 270.8	3.4	5.7	14.8	14.3

[a]Results are the mean of three experiments.
[b]Results are the mean of two experiments a day over a period of 5 days.

Table 3. Recovery of Indinavir, Saquinavir, and Ritonavir in Human Plasma

Compounds	Concentration (ng/mL)	Recovery (%± SD)[a]
Electrochemical detector		
Indinavir	200	90.0 ± 2.4
	300	86.0 ± 4.7
	400	92.0 ± 5.8
Saquinavir	200	95.0 ± 1.8
	300	90.0 ± 5.1
	400	98.0 ± 8.9
UV detector		
Saquinavir	200	97.0 ± 10.8
	300	96.0 ± 9.9
	400	89.0 ± 3.2
Ritonavir	1000	88.0 ± 0.03
	5000	89.0 ± 0.2
	10000	80.0 ± 0.3

[a]Results are the mean of three experiments.

Table 4. Protease Inhibitors Plasma Concentration in HIV-1 Infected Patients

Patient*	Plasma Concentration (ng/mL) UV	Plasma Concentration (ng/mL) ED
1	___	52.4 ± 9.0
2	22.0 ± 3.4	21.0 ± 4.0
3	___	11060 ± 42.0
4	27.0 ± 2.0	22.0 ± 3.0
5	___	75.1 ± 10.0
6	129.0 ± 7.0	130.0 ± 8.0
7	215.0 ± 7.0	218.0 ± 5.0
8	___	9850.0 ± 8.0
9	24.0 ± 10.0	31.0 ± 5.0
10	___	7270.0 ± 9.0
11	60.0 ± 3.0	50.0 ± 4.0
12	___	306.0 ± 10
13	___	1110 ± 4.0
14	___	220.0 ± 10.0
15	238.0 ± 2.0	277.0 ± 3.0
16	1185.0 ± 42.0	___
17	___	6155.0 ± 14.0
18	___	83.0 ± 1.0
19	___	13994.0 ± 970.0
20	220.0 ± 11.0	___

*Antiretroviral therapy is reported in Table 1.

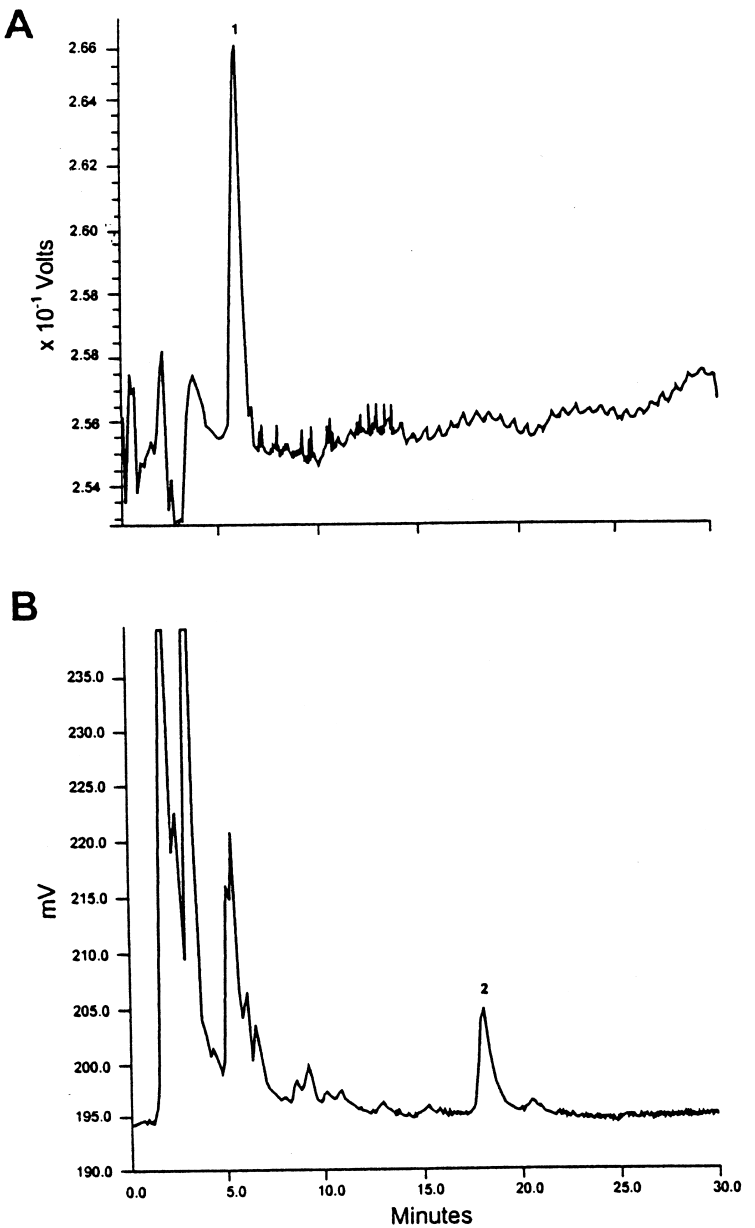

Figure 2. ED (A) and UV (B) Chromatograms obtained by injection of plasma samples containing 279.8 ng of Indinavir (1) and 23.8 ng of Ritonavir (2). Column and chromatographic conditions as described in Fig. 1.

Analysis of Plasma Samples

The applicability of the assay was demonstrated by analyzing plasma samples from HIV-1 infected patients receiving different protease inhibitors. The plasma concentrations of protease inhibitors in these patients, as determined by the currently described method, are shown in Table 4. Typical chromatograms are shown in Fig. 2.

The samples tested represent too few subjects to make useful observations relating to patient adherence and response to plasma antiretroviral concentration for these drugs

CONCLUSION

There are many reasons for the antiretroviral therapy failure including the acquisition or development of resistant viral strains, incomplete adherence to therapy, and pharmacokinatic reasons.(6,25) The latter are particularly important in relation to protease inhibitors, a class of drugs which are both metabolized by P450 isoenzymes, primarily CYP3A4, in the liver.(4) This results in considerable interindividual and intraindividual variability in plasma levels and a marked potential for drug interactions, leading to reduced(4)or elevated(26) drug concentration.

The method have described, permits analyzing three protease inhibitors simultaneously, using only 200 μL of plasma. It represents an efficient alternative to the systems currently found in the literature. Because of high individual variability of protease inhibitors metabolism, therapeutic drug level monitoring systems to optimize drug dosing may be useful in the future. Additional studies are under way to reach this aim.

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