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Simple and rapid high-performance liquid chromatographic method for nelfinavir, M8 nelfinavir metabolite, ritonavir and saquinavir assay in plasma

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Abstract

A simple reversed-phase liquid chromatographic method has been developed to determine protease inhibitors concentrations in plasma. Plasma samples (250 μl) containing protease inhibitors were prepared by a simple deproteinization (recovery: 92, 91, 91 and 90.5% for ritonavir, saquinavir, nelfinavir and M8 nelfinavir metabolite, respectively). Chromatography was accomplished using a Hypersil octadecylsilyl column (100×4.6 mm I.D.) and a mobile phase composed of acetonitrile, tetrahydrofuran and dihydrogenophosphate buffer (pH 4) (32:10:58, v/v). Ultraviolet detection at 210 nm was used. The limit of detection was 200 ng/ml for ritonavir, saquinavir, nelfinavir and M8 nelfinavir metabolite. Calibration curves were linear up to 20 000 ng/ml, with correlation coefficients better than 0.997 for all compounds. Intra- and inter-day coefficients of variation of the assay were ≤6% for all compounds. This method was used to analyse protease inhibitors plasma concentrations after oral administration within the framework of therapeutic drug monitoring and pharmacokinetic studies in AIDS patients.

Keywords: Nelfinavir; M8; Ritonavir; Saquinavir

1. Introduction

Protease inhibitors represent potent drugs for treatment of HIV disease. Plasma levels are required to ensure efficacy and to prevent treatment resistance [1–3]. Inter-patient pharmacokinetic variability of protease inhibitors is wide and does not always allow a sufficient drug exposure to maintain anti-viral activity [4–6]. Protease inhibitors inhibit the cytochrome P 450 to varying degrees [2,7–10], and a dual therapy with two protease inhibitors may lead to increased plasma concentrations of these drugs and antiretroviral efficacy [4,11]. Therefore, we decided to develop a simple protease inhibitors assay in

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plasma to apply next therapeutic drug monitoring (TDM) in all of the AIDS patients treated. The assay requirements were of a sufficient sensitivity and precision to allow pharmacokinetic studies, a short analysis time to get the plasma concentration results rapidly, and a small plasma sample volume to apply the method to paediatric patients.

A lot of high-performance liquid chromatography methods have already been developed for the determination of protease inhibitors in plasma. These methods used gradient [4,6,12–16] or isocratic elution [2,3,5,7,8,11,15–24], and one or several wavelengths for UV detection. For sample preparation, only two kinds of methods were found: liquid–liquid and solid–liquid extraction, frequently with addition of an internal standard.

Most published assays require costly equipment, with computer or diode array detector for example. All the methods described are time-consuming and do not give results rapidly. Therefore, we developed a HPLC assay of protease inhibitors (ritonavir, saquinavir, nelfinavir and its active metabolite M8) combining the advantages missing in the methods previously described, that is to say a very short analysis time due to a simplified sample treatment method and a small volume of plasma sample (250 μl).

2. Experimental

2.1. Chemicals

Nelfinavir and M8 nelfinavir metabolite were gifts from Agouron Pharmaceuticals (La Jolla, CA, USA), ritonavir from Abbott (Abbott Park, IL, USA), saquinavir from Roche Products (Welwyn Garden City, UK). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany), tetrahydrofuran (HPLC grade) from Prolabo (Fontenay-sous-Bois, France), methanol (HPLC grade) from SDS (Peypin, France), sterile distilled water Versol® from Aguettant (Lyon, France). Drug-free control serum Lyotrol N® was purchased from Biomérieux (Marcy l'Etoile, France). All other reagents were obtained from Merck.

2.2. HPLC apparatus or instrumentation

Liquid chromatographic apparatus was composed of a Shimadzu LC-6A pump, a Shimadzu C-R3A integrator/recorder, a Shimadzu SPD-6A UV–Vis detector, and a Shimadzu SIL-9A autoinjector from Touzart and Matignon (Courtaboeuf, France). The column used was a C_{18} Hypersil ODS column (3-μm particle size, 100×4.6 mm, from Touzart and Matignon). No pre-column was employed.

2.3. Chromatographic conditions

The separation of protease inhibitors was achieved with an isocratic solvent delivery system at a flow-rate of 1.2 ml/min at room temperature. The mobile phase was constituted of acetonitrile, tetrahydrofuran and a 20 m*M* potassium dihydrogenophosphate buffer adjusted to pH 4 with orthophosphoric acid (32:10:58, v/v), and was degassed daily by using ultrasonic.

Detection was performed at 210 nm for the three protease inhibitors and M8 metabolite.

The sample injection volume was 60 μl.

2.4. Standards

Individual stock solutions of each drug were prepared in methanol. These solutions were used to prepare a solution with ritonavir, saquinavir, nelfinavir and M8 metabolite at 100 μg/ml.

Protease inhibitors plasma standards were prepared by the following procedure: aliquots of drug-free plasma (Lyotrol N, Biomérieux) were spiked with 100 μg/ml protease inhibitors methanol stock solutions to obtain a first plasma solution containing 20 μg/ml of ritonavir or saquinavir or nelfinavir and M8 metabolite. This solution was further diluted with plasma to obtain the other concentrations: 0.2, 0.5, 1, 5, 10 μg/ml.

2.5. Sample treatment

A simple deproteinization procedure was developed, using only perchloric acid (35%) and methanol as reagents. Twenty-five μl of perchloric acid were added to a 250-μl aliquot of spiked plasma. The sample was mixed during 15 s exactly using a Top Mix 94323 vortex (Bioblock, Isle d'Abeau, France). Then 200 μl of methanol was added to each tube and vortexed during 1 min exactly. The sample were centrifuged for 15 min at 2000 *g* at 4 °C. 60 μl of the clear supernatant was injected onto the HPLC column for analysis at room temperature.

3. Results

3.1. Chromatographic separation

Retention times of M8 nelfinavir metabolite, ritonavir, saquinavir and nelfinavir were, respectively, 6.1, 8.1, 9.0 and 10.3 min (corresponding to k' of 6.51, 7.09, 8.03, 9.34, respectively). The drug-free plasma sample did not show any peak that would interfere with the analytes. Fig. 1 shows chromatograms of blank and spiked plasma. The resolution of protease inhibitors was 5.87 between M8 metabolite and ritonavir, 2.91 between saquinavir and ritonavir, and 3.61 between nelfinavir and saquinavir. Both are more than 2.5, allowing a quite correct separation. No endogenous compounds in plasma interfere with protease inhibitors. Many drugs able to be concomitantly administered with protease inhibitors during treatment were tested for specificity of the assay. Drug-free plasma samples were spiked separately with an appropriate concentration of each drug (therapeutic concentration) and were treated exactly the same as described above. Drugs (and concentrations assayed) were amprenavir (1 μg/ml), indinavir (1 μg/ml), acetaminophen (40 μg/ml), acetylsalicylic acid (140 μg/ml), amikacin (30 μg/ml), gentamycin (15 μg/ml), vancomycin (50 μg/ml), ranitidine (250 μg/ml), amitryptiline (50 μg/ml), aciclovir (20 μg/ml), ganciclovir (20 μg/ml), rifampicin (20 μg/ml), diazepam (20 μg/ml), tiemonium (20 μg/ml), amphotericin B (25 μg/ml), cotrimoxazole (30 μg/ml). These compounds showed different retention times from protease inhibitors in the chromatographic system or were not detected at the wavelength used. Amprenavir and indinavir were eluted at 4 and 4.5 min, respectively.

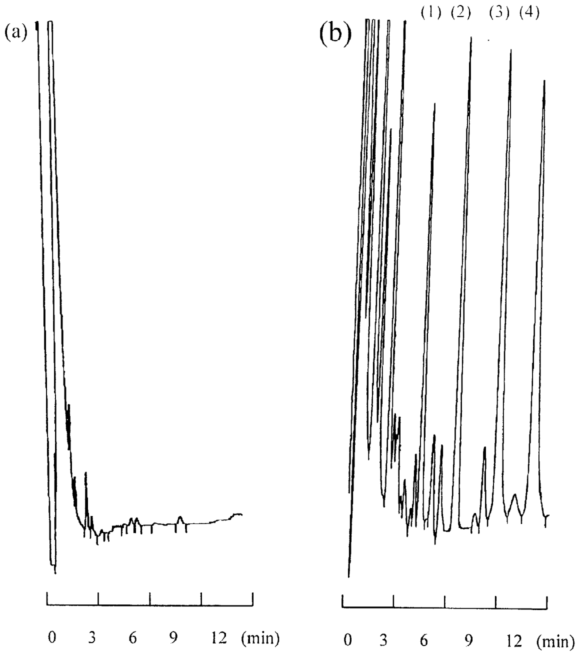

Fig. 1. (a) Chromatogram of blank human plasma and (b) chromatogram of a spiked human plasma with 2 μg/ml of M8 (1), ritonavir (2), saquinavir (3) and nelfinavir (4).

3.2. Quantitative analysis

Quantification was performed according to a linear least-square regression method and by plotting the peak height ratio against known concentrations of spiked plasma standards.

Drug-free plasma samples ($n=6$) spiked with ritonavir, saquinavir, nelfinavir and M8 nelfinavir metabolite (1 and 10 μg/ml) were treated according to the procedure described above and were compared with the same molecule and concentration in water samples (1 and 10 μg/ml) treated in the same conditions. Recovery was near 90% for all the protease inhibitors (for low and high concentration). Results are shown in Table 1.

The limit of quantification was defined as the lowest concentration in plasma sample such as the relative standard deviation between measured and nominal concentration was less than 20%. This limit of quantification was 200 ng/ml for all the analytes. The upper limit of quantification was arbitrarily

Table 1
Recovery (%) of the protease inhibitors assay

Theoretical concentration	Water ($n=6$)	Plasma ($n=6$)	Recovery (%)
Nelfinavir			
1	1.13	0.97	86.1
10	10.38	9.90	95.4
M8 Nelfinavir metabolite			
1	1.17	0.99	84.6
10	10.59	10.25	94.4
Saquinavir			
1	1.15	1.00	86.6
10	10.39	9.92	95.5
Ritonavir			
1	1.15	1.07	93.0
10	10.67	9.71	91.1

defined at 20 μg/ml. This limit was largely above the current plasma concentrations found in humans.

Linearity was perfect for ritonavir ($r=0.997$, $P<0.001$), saquinavir ($r=0.999$, $P<0.001$), nelfinavir ($r=0.998$, $P<0.001$), and M8 nelfinavir metabolite ($r=0.999$, $P<0.001$) with plasma concentrations ranging from 200 to 20 000 ng/ml.

3.3. Accuracy and precision

The intra-day variability of the assay was tested on six samples of two different concentrations of the three protease inhibitors ritonavir, saquinavir, nelfinavir and M8 nelfinavir metabolite in plasma, all prepared on the same day. These samples were also used to test the assay accuracy. The inter-day variability of the assay was tested for two different concentrations of protease inhibitors in plasma, for six separate days. Results of precision and accuracy are presented in Table 2.

3.4. Clinical applications

The chromatographic assay described here has been applied to therapeutic drug monitoring in patients treated by one or other of the proteases of interest. Fig. 2 shows the chromatograms of a patient receiving nelfinavir (Viracept®, 1250 mg/12 h) and a patient receiving saquinavir (Invirase®, 600 mg/8 h), combined to others antiretroviral agents. No interference with endogenous compounds or co-medication or other metabolites was noted.

Table 2
Accuracy and precision of the protease inhibitors assay

Assay	Theoretical concentration (μg/ml)	*n*	Mean measured concentration (μg/ml)	C.V. (%)	Accuracy (%)
Intraday:					
Ritonavir	1	6	1.005	4.47	100.5
	10	6	9.744	1.67	97.4
Saquinavir	1	6	1.042	1.59	104.2
	10	6	10.070	3.95	100.7
Nelfinavir	1	6	1.010	4.91	101.0
	10	6	10.580	3.13	105.8
M8 metabolite	1	6	1.007	2.98	100.7
	10	6	10.195	3.14	101.9
Interday:					
Ritonavir	1	6	1.060	1.90	106.0
	10	6	9.736	0.70	97.4
Saquinavir	1	6	1.023	4.93	102.3
	10	6	9.835	1.43	98.4
Nelfinavir	1	6	1.006	5.33	100.6
	10	6	9.819	1.41	98.2
M8 metabolite	1	6	0.993	2.88	99.3
	10	6	10.253	2.41	102.5

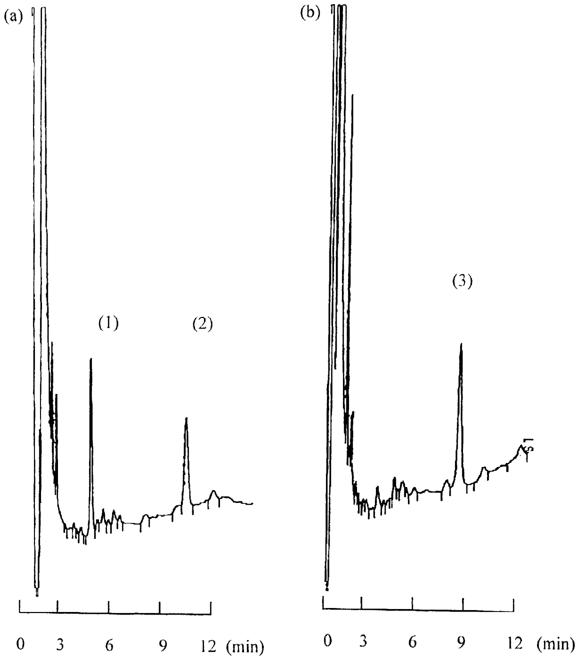

Fig. 2. (a) Chromatogram of a patient sample containing 712 ng/ml of M8 metabolite (1) and 1468 ng/ml of nelfinavir (2) and (b) chromatogram of a patient sample containing 823 ng/ml of saquinavir (3).

4. Discussion

The above-described method is the most rapid HPLC assay for protease inhibitors determination in plasma. Sample treatment and chromatographic analysis require only 15 and 14 min, respectively. In fact, one simple step for sample preparation (deproteinization) was considerably simplified with regards to other methods [9–16,28]. Only two reagents were employed for a one-step sample preparation. Solid-phase extraction or liquid–liquid extraction were not used and appeared not to be necessary. In addition, this method needs only simple chromatographic apparatus (isocratic elution, monochromatic UV detection), available in all hospital laboratories. Moreover, despite a very simplified sample treatment, the assay remains quite sensitive. These conditions make it quite suitable for high-return therapeutic drug monitoring, when there is a need to get results rapidly. Also, it is particularly easy to perform since no special equipment is needed.

Existing HPLC methods do not present simultaneously both the characteristics required for paediatric patients, such as a small volume of plasma sample (250 μl), and pharmacokinetic studies such as a high precision and high sensibility. Few methods require small sample volume [8,19,23,24], others require 500 μl or more [3,6,9,11–13,15–18,21,28].

Most of the existing HPLC methods used acetonitrile for plasma deproteinization. We preferred to use methanol rather than acetonitrile, because the solubility of protease inhibitors is enhanced in methanol. All these precautions contribute to enhance reproducibility and sensitivity. Despite the method developed here being less sensitive than other ones, the quantification limit is quite sufficient for therapeutic drug monitoring and pharmacokinetic studies. However, accuracy and precision (C.V.<6%) are better than a lot of published methods [7,11,16–18,23,24] which present coefficients of variation up to 15%. The other methods used liquid–liquid extraction or solid-phase extraction process. These techniques are more time-consuming than a simple deproteinization step and required qualified staff. The lack of heavy extraction procedure and the good precision obtained exempted us from using an internal standard. This represents another advantage, since no additional compound has to be separated from the antiproteases by the chromatographic system. In addition, no precolumn was necessary because the step of deproteinization was sufficient to delete endogenous compounds of plasma which could decrease the column performance.

In summary, this method provides several advantages over all existing methods. However, it was not possible to separate all the protease inhibitors marketed at this date (others are indinavir, amprenavir, tipranavir and lopinavir) in a single run under the chromatographic conditions described above, since the separation of several molecules required a more complex equipment. Despite published methods are able to quantify up to six protease inhibitors in one analytical run, they will not be valuable for all the upcoming new potent protease inhibitors [25–28]. Moreover, it seems difficult to separate simultaneously six or more protease inhibitors in one analytical run. Only one method was described with six proteases inhibitors but presents a long analysis time [27,28]. For routine therapeutic drug monitoring practice, it seems more suitable to use a simple sample treatment and get results quickly, since only two or three protease inhibitors are simultaneously administered in a same patient.

Therapeutic monitoring of protease inhibitors is widely recommended since low plasma concentrations can lead to treatment resistance or failure. This simple and easy to perform method could allow an increase in the number of monitored patients.

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