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Short communication

Simultaneous high-performance liquid chromatographic determination of the antiretroviral agents amprenavir, nelfinavir, ritonavir, saquinavir, delavirdine and efavirenz in human plasma

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Abstract

This article describes a method for the simultaneous determination of four licensed HIV protease inhibitors (amprenavir, nelfinavir, saquinavir and ritonavir) and two novel non-nucleoside reverse transcriptase inhibitors (efavirenz and delavirdine) in human plasma in a single run. Plasma samples (500 μl) were treated by liquid–liquid extraction with methyl *tert*.-butyl ether. The compounds were separated by reversed-phase liquid chromatography on a C_{18} column with spectrophotometric detection at 260 nm. The method is linear over the specific ranges investigated, accurate (inaccuracy <11.7%) and showed intra-day and inter-day precision within the ranges of 0.9–7.0 and 1.9–8.8%, respectively. The six compounds were stable in plasma after 6 months of storage at −20°C and after five freeze–thaw cycles.

Keywords: Amprenavir; Nelfinavir; Ritonavir; Saquinavir; Delavirdine; Efavirenz

1. Introduction

The development of a large number of new antiretroviral agents and the increasing resistance of HIV virus has led to changes in the treatment of this infection. Clinicians prescribe for this a large number of possible combinations of several antiretroviral drugs. Therapeutic monitoring of these drugs is recommended in order to avoid or to delay resistance from the virus, to avoid the usually underestimated non-adherence and to manage drug interactions. In view of this, analytical methods have already been described to quantify single [1–12] and combined [13–16] anti-HIV agents in biological media.

We have developed a new method to analyse six antiretroviral drugs in one analytical run. These agents include: four licensed HIV protease inhibitors (amprenavir, nelfinavir, saquinavir and ritonavir) and two novel non-nucleoside reverse transcriptase inhibitors (efavirenz and delavirdine). This simple method allows researchers to save time and decrease cost compared with that of six different assays. We report here the development and validation of a

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high-performance liquid chromatography (HPLC) assay with UV detection for simultaneous quantitative determinations of these six antiretroviral agents in human plasma.

2. Experimental

2.1. Equipment

The liquid chromatographic equipment consists of a WISP 717+ automatic sample injector (Waters, Millipore, Saint Quentin, France), a Shimadzu LC 10 AD pump (Touzart & Matignon, Les Ulis, France), and a Shimadzu SPD 10 AVP spectrophotometric detector programmed at 260 nm (Touzart & Matignon). Data were recorded and analyzed with a Class VP automated software system (Touzart & Matignon). Separation of the six antiretroviral agents was performed on a Lichrospher 100 RP-18 (5 μm) (Lichrocart 125-4 HPLC cartridge) with a Lichrospher 100 RP-18 (5 μm) (Lichrocart 4-4) guard column (Merck, Darmstadt, Germany).

2.2. Chemicals

Amprenavir, nelfinavir mesylate, ritonavir, saquinavir mesylate and delavirdine mesylate were gifts from Glaxo Wellcome (Hertfordshire, UK), Agouron Pharmaceuticals (La Jolla, CA, USA), Abbott (North Chicago, IL, USA), Hoffmann Laroche (Basel, Switzerland) and Pharmacia Upjohn (Saint Quentin, France), respectively. As pure efavirenz powder was not directly available from the manufacturer, it was extracted from the commercialized Sustiva formulation by 10 repeated extractions with 10 ml methanol. Completion of the extraction was controlled by determining efavirenz in the methanolic fractions and by comparing our solution with a titrated efavirenz solution prepared from pure efavirenz kindly supplied by Anne Marie Taburet (Bicetre Hospital, Kremlin Bicetre, France). The internal standard [6,7-dimethyl-2,3-di-(2-pyridyl)-quinoxaline] was purchased from Sigma–Aldrich (Saint-Quentin, France).

Acetonitrile, diethylamine and methyl *tert*.-butyl ether were from Mallinckrodt Baker (Noisy le Sec, France). Hydrochloric acid 36% and tetrahydrofuran were from Osi (Elancourt, France). Orthophosphoric acid 85%, ethanol and methanol were from Prolabo (Fontenay-sous-Bois, France).

2.3. Chromatographic conditions

Chromatographic analyses were performed at room temperature and at a flow-rate of 0.5 ml/min with a mobile phase composed of sodium phosphate 25 m*M*–acetonitrile (55.2:44.8, v/v) modified with diethylamine 0.9% and tetrahydrofuran 1% with the pH adjusted to 3.0 with orthophosphoric acid. The mobile phase was ultrasonicated and filtered.

2.4. Preparation of standards

Stock solutions of amprenavir, efavirenz, nelfinavir, ritonavir, saquinavir and the internal standard were prepared by dissolving the appropriate amount of drug in methanol to a final drug concentration of 1000, 1000, 400, 100, 1000 and 200 μg/ml, respectively. Stock solution of delavirdine was prepared by dissolving the appropriate amount of drug in a mixture of acetonitrile–methanol (1:9, v/v) to a final concentration of 1000 μg/ml. These stock solutions were each stable for at least 6 months at −20°C. Working solutions were prepared by diluting these stock solutions in methanol, and were used immediately for the preparation of quality control samples and calibration samples. A working solution of the internal standard was prepared by dissolving the stock solution in methanol to a concentration of 4 μg/ml.

2.5. Sample treatment

To 500 μl of plasma (patient sample, quality control or calibration standard) were added 100 μl of the internal standard solution (4 μg/ml in methanol) and 250 μl of sodium hydroxide 0.1 *N* adjusted to pH 10 with hydrochloric acid. The mixture was vortex-mixed for 1 min. Four millilitres of methyl *tert*.-butyl ether were added to the mixture. Tubes were gently and horizontally shaken for 20 min prior to being centrifuged at 3000 rpm at +4°C. The aqueous phase was frozen using dry ice in ethanol. The organic phase was decanted and evaporated under a gentle stream of nitrogen at 37°C. The

residue was then reconstituted in 150 μl of mobile phase and 100 μl were injected.

To avoid any adsorption on glass, all extraction procedures were carried out using Vacutainer siliconed glass tubes (Beckton Dickinson, Plymouth, UK).

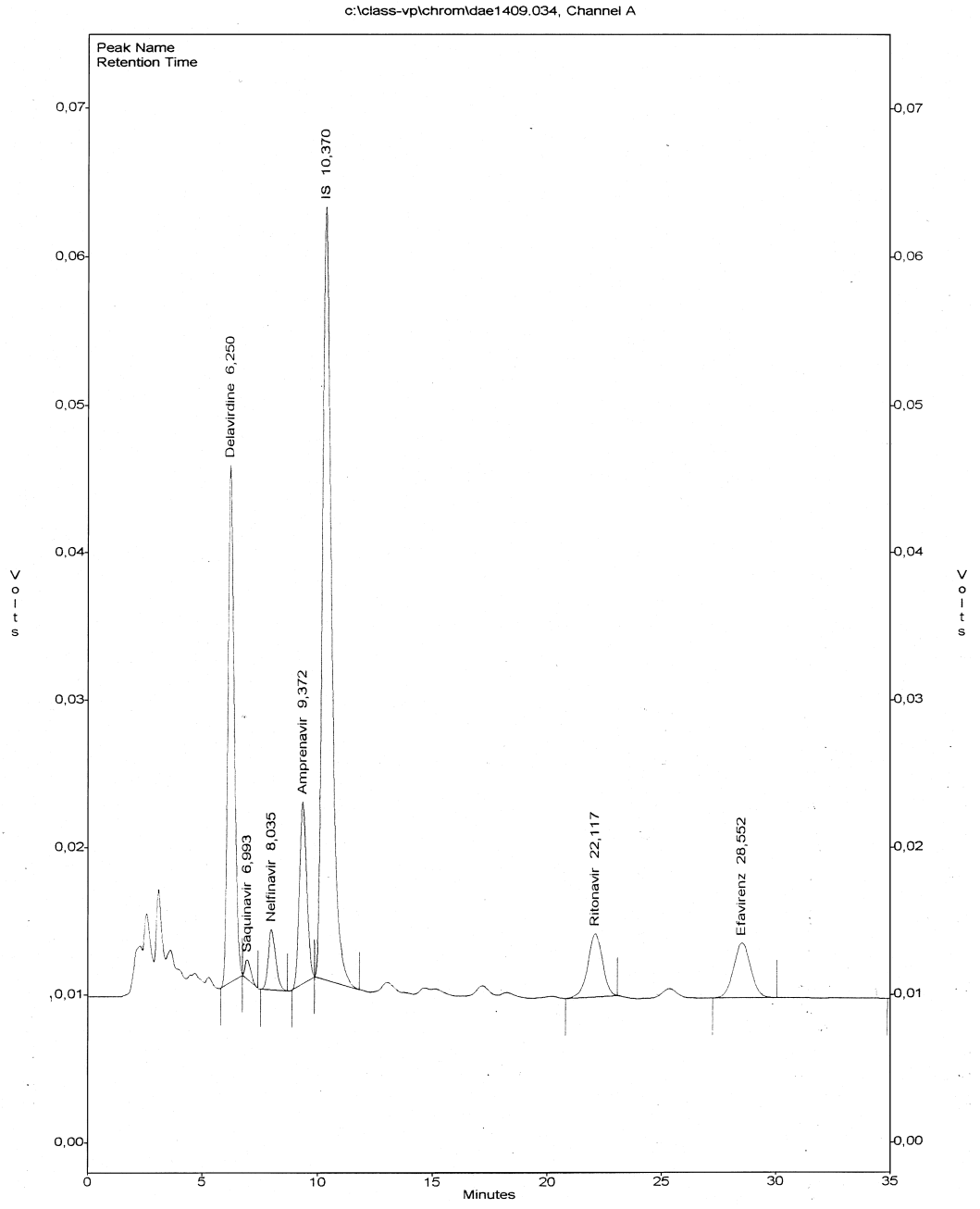

Fig. 1. Chromatogram of a plasma spiked with 500 ng/ml amprenavir, 1000 ng/ml nelfinavir, 2500 ng/ml ritonavir, 600 ng/ml saquinavir, 1250 ng/ml delavirdine, 625 ng/ml efavirenz and 100 μl of a 4 μg/ml methanolic solution of internal standard (IS).

2.6. Specificity

Specificity was investigated by analyzing compounds for interference in plasma spiked with possible co-administered drugs. These drugs included: zidovudine, ddI, ddC, d4T, 3TC, indinavir, nevirapine, abacavir, aciclovir, ganciclovir, ciprofloxacine, diazepam, bromazepam, prazepam, fluoxetine, clomipramine, cimetidine, ranitidine, acetaminophen, sulfamethoxazole, trimetoprime, amikacin, amoxicilline, rifampicin, ethambutol, isoniazide, amphotericin B, caffeine, aspirin, ibuprofen, phenobarbitone, diphenylhydantoine and clomipramine. Each of these drugs was tested at therapeutic concentrations.

2.7. Linearity

Standard curves were determined by spiking drug-free plasma with the six compounds at the following concentration ranges: amprenavir (0–8000 ng/ml), nelfinavir (0–10 000 ng/ml), ritonavir (0–16 000 ng/ml), saquinavir (0–2000 ng/ml), delavirdine (0–20 000 ng/ml) and efavirenz (0–8000 ng/ml). Seven spiked plasma samples were analyzed for the construction of each calibration curve. For the six antiretroviral agents, linearity of three calibration curves was tested with the F-test for lack of fit.

2.8. Precision and accuracy

Five samples of two quality controls at low and high concentrations were analyzed for the calculation of the coefficient of variation and were carried out for intra-day and inter-day validations. Accuracy was calculated as the percentage of the nominal concentration.

2.9. Recovery

The recovery of the method was determined by comparing the peak areas of the samples at four

Table 1
Intra-day and inter-day precision for the determination of amprenavir, nelfinavir, ritonavir, saquinavir, delavirdine and efavirenz in human plasma

	Intra-day validation			Inter-day validation		
	Spiked plasma conc. (ng/ml)	Mean determined conc. (ng/ml)	CV (%) ($n = 5$)	Spiked plasma conc. (ng/ml)	Mean determined conc. (ng/ml)	CV (%) ($n = 15$)
Amprenavir						
High	6000	5769	2.7	6000	5837	2.2
Low	150	138	1.0	150	142	8.8
Nelfinavir						
High	5000	5393	3.2	5000	5266	4.6
Low	500	521	7.0	500	524	5.5
Ritonavir						
High	8000	7599	4.0	8000	8058	6.5
Low	2000	2091	2.8	2000	2066	4.1
Saquinavir						
High	1500	1460	3.5	1500	1568	6.2
Low	500	552	3.5	500	510	7.9
Delavirdine						
High	15 000	14 859	1.9	15 000	14 828	1.9
Low	500	480	3.4	500	465	7.5
Efavirenz						
High	6000	6191	0.9	6000	6034	4.6
Low	750	731	1.4	750	768	6.0

concentrations after extraction with the peak areas of standard solutions at the same concentrations.

2.10. Limit of quantification

Plasma samples were spiked with decreasing concentrations of the studied compounds and analyzed. The limit of quantification was considered to be the lowest concentration that could be measured with a coefficient of variation less than 25%.

2.11. Stability

A batch of calibration samples and control samples was submitted to five freeze–thaw cycles and analyzed. Another batch of calibration and control samples was stored at −20°C for 6 months and analyzed.

2.12. Calculation procedures for determination of quality controls and patient samples

Daily calibration curves were constructed using the ratios of the observed peak area of each compound to its internal standard. For quality controls and patient samples, the unknown concentrations were computed from the linear regression equation of the peak area ratio against concentration for the calibration curve.

2.13. Analysis of patient samples

Blood was collected on sodium heparin. Plasma was separated by centrifugation at 3000 rpm for 5 min at +4°C and was immediately stored at −20°C until analysis.

Table 2
Inaccuracy for the determination of amprenavir, nelfinavir, ritonavir, saquinavir, delavirdine and efavirenz in human plasma

	Theoretical plasma conc. (ng/ml)	Observed plasma conc. (ng/ml)	Inaccuracy (%) ($n = 5$)
Amprenavir			
Low	200	194	2.9
Medium	1000	947	5.3
High	7000	6866	1.9
Nelfinavir			
Low	800	755	5.6
Medium	2000	1767	11.7
High	8000	7230	9.6
Ritonavir			
Low	1500	1460	2.7
Medium	5000	5163	3.3
High	10 000	9422	5.8
Saquinavir			
Low	400	390	2.5
Medium	1200	1119	6.7
High	2000	1850	7.5
Delavirdine			
Low	400	384	4.1
Medium	2500	2443	2.3
High	17 500	17 928	2.4
Efavirenz			
Low	500	498	0.4
Medium	1250	1167	6.6
High	7000	6852	2.1

3. Results and discussion

Fig. 1 shows a chromatogram of a plasma sample spiked with the six compounds. Approximate retention times and the corresponding variabilities of the six studied antiretroviral agents observed on two different column batches were 9.4, 8.0, 22.2, 7.0, 6.3 and 28.6 min for amprenavir, nelfinavir, ritonavir, saquinavir, delavirdine and efavirenz, respectively. No interference occurred with the anti-HIV drugs that can be co-administered with the six studied compounds. Retention times were similar for fluoxetin and delavirdine (5.9 and 6.3 min, respectively) and for clomipramine, saquinavir and nelfinavir (7.5, 7.0 and 8.0 min, respectively). Fluoxetin and clomipramine were tested at peak concentrations found in therapeutics (500 and 80 ng/ml, respectively) and did not interfere with the determination of antiretroviral agents.

The method showed good intra-day and inter-day precision with coefficients of variation (CVs) ranging from 0.9 to 7.0 and from 1.9 to 8.8%, respectively (Table 1). Analyses were linear over the ranges investigated and proved to be accurate with an average inaccuracy of less than 11.7% (Table 2). The extraction recoveries were 90.4, 93.9, 95.2, 98.1, 86.4 and 83.8% for amprenavir, nelfinavir, ritonavir, saquinavir, delavirdine and efavirenz, respectively. The internal standard was extracted with a recovery of 83.8%.

Limits of quantification were 50, 150, 100, 100, 75 and 75 ng/ml for amprenavir, nelfinavir, ritonavir, saquinavir, delavirdine and efavirenz, respectively. The limit of quantification of saquinavir is high if administered alone, but adequate if co-administered with another HIV protease inhibitor such as ritonavir.

The six compounds were stable in plasma under the different storage conditions (five freeze–thaw cycles) and treatment.

In conclusion, this rapid, specific and validated assay can be used to monitor six antiretroviral agents in human plasma in a single run.

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