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Determination of 19 antiretroviral agents in pharmaceuticals or suspected products with two methods using high-performance liquid chromatography

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Abstract

Three classes of antiretroviral agents are usually available for the treatment of HIV infection: nucleoside reverse transcriptase inhibitors (IN), non-nucleoside reverse transcriptase inhibitors (INN) and protease inhibitors (IP). Two methods by reversed-phase liquid chromatography were developed for the analysis of 19 antiretroviral molecules belonging to these three therapeutic classes and used in medicinal products. Both of these HPLC techniques use a C18 column and UV detection. The first method is for IN family analysis and allows eight molecules to be separated: zalcitabine, lamivudine, amdoxovir, emtricitabine, didanosine, stavudine, zidovudine and abacavir. The second method is for INN and IP family analysis and allows 11 molecules to be separated: fosamprenavir, nevirapine, indinavir, amprenavir, saquinavir, atazanavir, ritonavir, lopinavir, efavirenz, nelfinavir and tipranavir. The combination of these two methods makes possible the quality control of mono-, bi- or tri-therapy pharmaceutical products and the detection of illegal products sold particularly in developing countries.

Keywords: HIV; Antiretroviral agent; Nucleoside reverse transcriptase inhibitors; Non-nucleoside reverse transcriptase inhibitors; Protease inhibitors; HPLC–UV; Illegal products

1. Introduction

Availability of good quality medicines is of major importance both in developed and developing countries. Counterfeit of essential medicines, such as antibiotics, antimalarials and antiretrovirals, is a public health danger that can lead to life threatening situations [1,3]. In such a context and in order to make available screening methods for suspected products, a methodology has been developed to assess the quality of most antiretroviral medicines available in 2006 for HIV treatment.

In order to check medicine quality and, in particular, generic specialities distributed in developing countries, the aim of our laboratory was to develop a protocol for the identification and quantification of the most antiretroviral molecules available in the international market. These molecules belong mainly to three therapeutic families [2]: nucleoside/nucleotide reverse transcriptase inhibitors (IN), non-nucleoside reverse transcriptase inhibitors (INN) and protease inhibitors (IP). Researching in scientific literature [4–15] and compiling a library of antiviral molecules led us to develop and validate two complementary HPLC methods (on C18 column with solvent gradient and UV detection). The first method allowed eight IN family molecules to be separated: zalcitabine, lamivudine, amdoxovir, emtricitabine, didanosine, stavudine, zidovudine and abacavir. The second method allowed 11 molecules belonging to the INN and IP families to be separeted: fosamprenavir, nevirapine, indinavir, amprenavir, saquinavir, atazanavir, ritonavir, lopinavir, efavirenz, nelfinavir and tipranavir. For active ingredient verification and quantification, these methods were designed to be as easy as possible to work.

2. Experimental

2.1. Reagents

Standards and compounds were kindly obtained from the respective pharmaceutical companies: atazanavir sulfate, didanosine, efavirenz and stavudine from Bristol Myers Squib

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(Epernon, France); zidovudine, lamivudine, abacavir sulfate, amprenavir and fosamprenavir calcic from GlaxoSmithKline (Greenford, UK); amdoxovir, emtricitabine and tenofovir disoproxil fumarate from Gilead (Paris, France); enfuvirtide, nelfinavir mesylate, saquinavir mesylate and zalcitabine from Roche (Neuilly-sur-seine and Fontenay-sous-bois, France); indinavir sulfate from Merck Charp & Dohme–Chibret (Clermont-Ferrand, France), lopinavir and ritonavir form Abbott (Saint-Rémy-sur-Avre, France); nevirapine anhydre, nevirapine hemihydrate and tipranavir from Boeringer Ingelheim (Paris, France).

Acetonitrile (Carlo Erba, France) and methanol (BDH, France) were HPLC grade. Ammonium acetate, potassium dihydrogenphosphate (KH_2PO_4), acetic acid 100% (Prolabo, France), potassium hydroxide 37% (Carlo Erba, France) were analytical-reagent grade. Water was ultra pure HPLC grade (Milli-Q, Millipore).

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a Waters model composed of a 600 quaternary pump, a 996 diode array detector and a Wisp 717+ autosampler injector operated with the Millenium 32 software.

The first HPLC method enabled IN molecules identification and assay. The separation was performed using an YMC-pack ODS-AM, 250 × 4.6 mm, 5 μm analytical column (Interchim, France). The mobile phase was composed of an ammonium acetate buffer (25 mM adjusted to pH 4.0 with acetic acid 100%) filtered through a 0.45 μm polypropylene membrane (GHP, Pall Gelman) and ultrasonically degassed for 15 min. A gradient with ammonium acetate buffer and methanol was programmed. The proportion of methanol stayed at 5% for 5 min, rose to 30% in 25 min and stayed at 30% for 15 min before falling back down to 5% in 5 min. The system was then equilibrated for 10 min under initial conditions. The mobile phase was delivered at a flow rate of 1 ml/min. The sample injection volume was 10 μl.

The second HPLC method enabled INN and IP molecules identification and assay. The separation was performed using a Symmetry C18 250 × 4.6 mm, 5 μm column (Waters, France). The mobile phase was composed of potassium phosphate buffer (50 mM adjusted to pH 5.65 with potassium hydroxide 100%) filtered through a 0.45 μm polypropylene membrane (GHP, Pall Gelman) and ultrasonically degassed for 15 min. A gradient with the potassium phosphate buffer and acetonitrile was programmed. The proportion of acetonitrile stayed at 40% for 5 min, rose to 60% in 35 min and stayed at 60% for 5 min before falling back down to 40% in 1 min. The system was then equilibrated for 4 min under initial conditions. The mobile phase was delivered at a flow rate of 1.5 ml/min. The sample injection volume was 20 μl. A summary of the two methods is reported in Table 1.

2.3. Calibration curves preparation

Substance solubilities were determined. Standards for IN family molecules were dissolved in water. Standards for INN and IP family molecules were dissolved in methanol, except for atazanavir sulphate which is water soluble and fosamprenavir calcic which is soluble in acidified water. Enfuvirtide, belonging to a fourth family (entry inhibitors), was used to study possible interferences. This molecule is soluble in dimethylformamide.

Stock solutions of IN family molecules were prepared in water at a 0.5 mg/ml concentration. A calibration range was carried out in a dilution solvent (methanol 5%–water 95%) in order to obtain concentrations of 10, 25, 50, 75 and 100 μg/ml for each compound. Stock solutions of INN and IP family molecules were prepared in methanol at the concentration of 0.5 mg/ml. A calibration range was carried out in order to obtain concentrations

Table 1
Summary of HPLC methods

	Method 1			Method 2		
Column	YMC pack ODS-AM, 250 × 4.6 mm, 5 μm			Symmetry C18 250 × 4.6, 5 μm		
Mobile phase A	Ammonium acetate buffer 0.025 M pH 4.0			Potassium phosphate buffer 50 mM pH 5.65		
Mobile phase B	Methanol			Acetonitrile		
Gradient	Time (min)	%A	%B	Time (min)	%A	%B
	0	95	5	0	60	40
	5	95	5	5	60	40
	30	70	30	40	40	60
	45	70	30	45	40	60
	50	95	5	46	60	40
	60	95	5	50	60	40
Flow rate	1.0 ml/min			1.5 ml/min		
UV detection	λ = 270 nm			λ = 260 nm		
Injection volume	10 μl			20 μl		
Column temperature	Ambient			30 °C		
Run time	60 min			50 min		
Dissolve solvent	Milli-Q water			Methanol		
Diluents	Milli-Q water 95%–methanol 5%			Initial mobile phase		

of 10, 25, 50, 75 and 100 μg/ml for each molecule. The initial mobile phase (acetonitrile 40%–ammonium acetate buffer 60%) was used as dilution solvent.

2.4. Sample preparation

The sample weight (equivalent to two or four tablets or capsules accurately weighed) was placed directly into a graduated flask with a little water, and it underwent magnetic stirring for 30 min. The preparation was then diluted to volume with water for medicine containing IN family molecules or diluted with methanol for medicine containing INN and IP family molecules. The solution was centrifuged for 15 min at 4000 rotation/min. Supernatant dilutions were then performed with the methanol 5%–water 95% mixture for the first family and with the mobile phase (acetonitrile 40%–ammonium acetate buffer 60%) for the two other families in order to obtain approximately 50 μg/ml sample solutions. Solutions were filtered by acrodisk GHP 0.45 μm (Pall Gelman) before injection. It should be noted that a test has shown that acrodisk filtered solutions did not modify studied peak areas.

3. Results

3.1. Specificity and selectivity

UV spectra of IN family molecules differ from each other and are compound specific, except for zalcitabine and lamivudine, and stavudine and zidovudine for which spectra are similar. Maximum absorption ranges between 250 and 290 nm. A 270 nm wavelength was chosen for the analysis, being appropriate for whole molecules determination in the selected concentration range. UV spectra of INN and IP family molecules also differ from each other and are compound specific, except for fosamprenavir and amprenavir for which spectra are similar. Maximum absorption ranges between 240 and 280 nm. A 260 nm wavelength was chosen for the analysis, being appropriate for whole molecules determination in the selected concentrations range. The use of a photodiode array detector allowed, on one hand, antiretroviral molecule identification by comparison with the reference spectrum and on the other hand, the detection of other non-antiretroviral molecules added to the speciality in case of falsification.

There is no interference of IN family molecules during the analysis of INN and IP family molecules, and vice versa. The study was supplemented by injecting three additional compounds which did not cause any interference: enfuvirtide (entry inhibitor antiretroviral), hypoxanthine (degradation product of didanosine) and aciclovir (antiviral). Analysing over 110 medicinal products enabled the verification of the absence of interference of excipients commonly used in tablet and capsule formulation. Symmetry factor and resolution were calculated for each peak in both chromatographic systems studied, in accordance to the recommendations of the European Pharmacopeia 5th edition. Results are reported in Tables 2 and 3. The symmetry factor always complied with the recommendations of the European Pharmacopeia (between 0.8 and 1.5) and the resolution was always higher than 1.5 (minimal resolution for a complete separation).

Table 2
Chromatographic parameters of method 1 for IN family

Method 1 for IN			
Name	t_R (min)	Symmetry	Resolution
Zalcitabine	14.1	1.0	–
Lamivudine	18.2	1.0	12.7
Amdoxovir	20.1	1.1	6.9
Emtricitabine	23.4	1.1	12.3
Didanosine	24.0	1.1	2.3
Stavudine	24.4	1.0	1.5
Zidovudine	37.3	1.0	44.9
Abacavir	44.2	1.1	18.4

3.2. Concentration range

The study of method linearity was carried out on 10–25–50–75 and 100 μg/ml standard solutions, and the study of reliability was carried out on a 50 μg/ml standard solution (Figs. 1–4).

3.3. Linearity and precision

Validation was done according to recommendations [16,17] and using AVA software (3rd version). The statistical study applied to the eight IN compounds (Table 4) led to the following conclusions on method n°1:

- variances in linearity and precision were homogeneous for the three series of injections (carried out on three different days),
- the correlation coefficient of the linear calibration curve was always greater than 0.996,
- the linearity of the calibration curve was shown (statistical tests of variance homogeneity, comparison of *y*-intercept with 0, existence of a significant slope and validity of the linear curve are in compliance),
- the relative standard deviation of injections, repeatability and intermediate precision were always less than 1.2% (except for Didanosine, a fragile molecule in solution which presented an intermediate precision RSD of 5.7%).

Table 3
Chromatographic parameters of method 2 for INN and IP families

Method 2 for INN and IP			
Name	t_R (min)	Symmetry	Resolution
Fosamprenavir	2.3	1.2	–
Nevirapine	2.7	1.3	2.8
Indinavir	6.7	1.2	18.1
Amprenavir	11.5	1.2	14.0
Saquinavir	19.8	1.1	20.6
Atazanavir	20.7	1.1	2.2
Ritonavir	23.0	1.1	5.3
Lopinavir	25.6	1.1	5.9
Efavirenz	27.2	1.1	3.5
Nelfinavir	34.2	1.0	13.6
Tipranavir	39.5	1.1	8.2

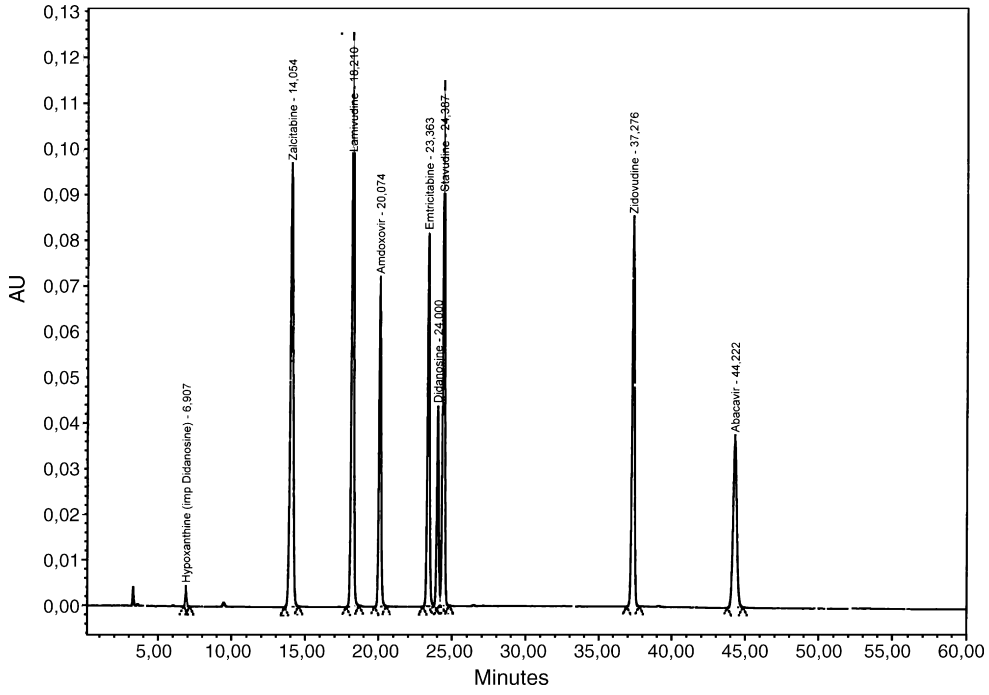

Fig. 1. Method 1 chromatogram (IN family).

The statistical study applied to the 11 INN and IP compounds (Table 5) allowed the following conclusions to be drawn on method n°2:

- variances in linearity and precision were homogeneous for the three series of injections (carried out on three different days),
- the correlation coefficient of the linear calibration curve was always greater than 0.999,
- the linearity of the calibration curve was shown (statistical tests of variance homogeneity, comparison of *y*-intercept with 0, existence of a significant slope and validity of the linear curve are in compliance),
- the relative standard deviation of injections, repeatability and intermediate precision were always less than 1.0% (except for Lopinavir which presented a RSD of 2% because of low absorbance at maximum absorption $\lambda = 260$ nm).

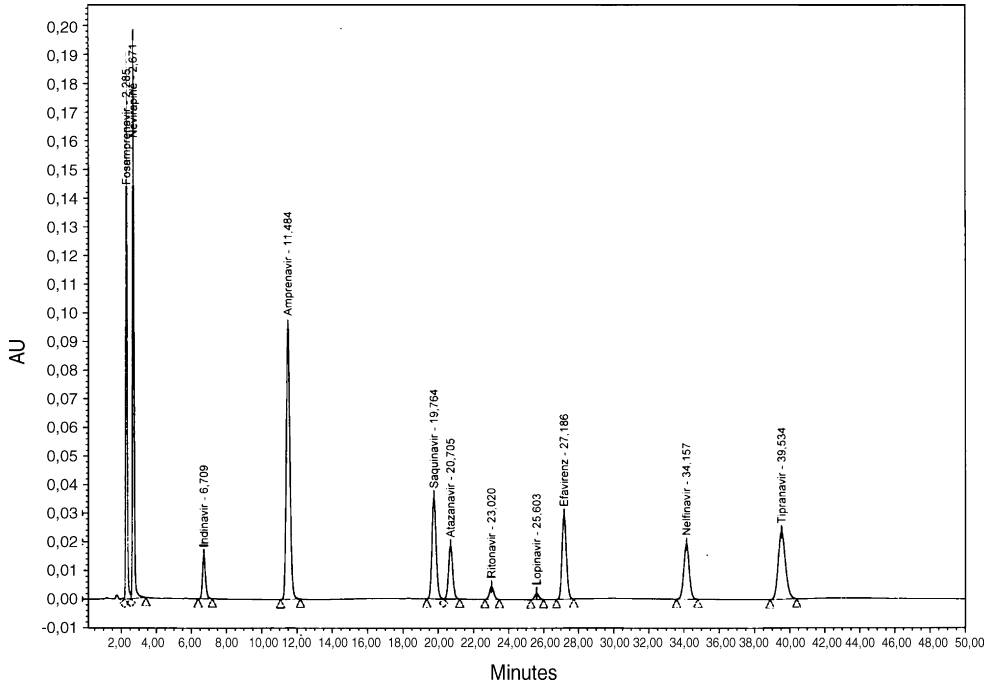

Fig. 2. Method 2 chromatogram (INN and IP families).

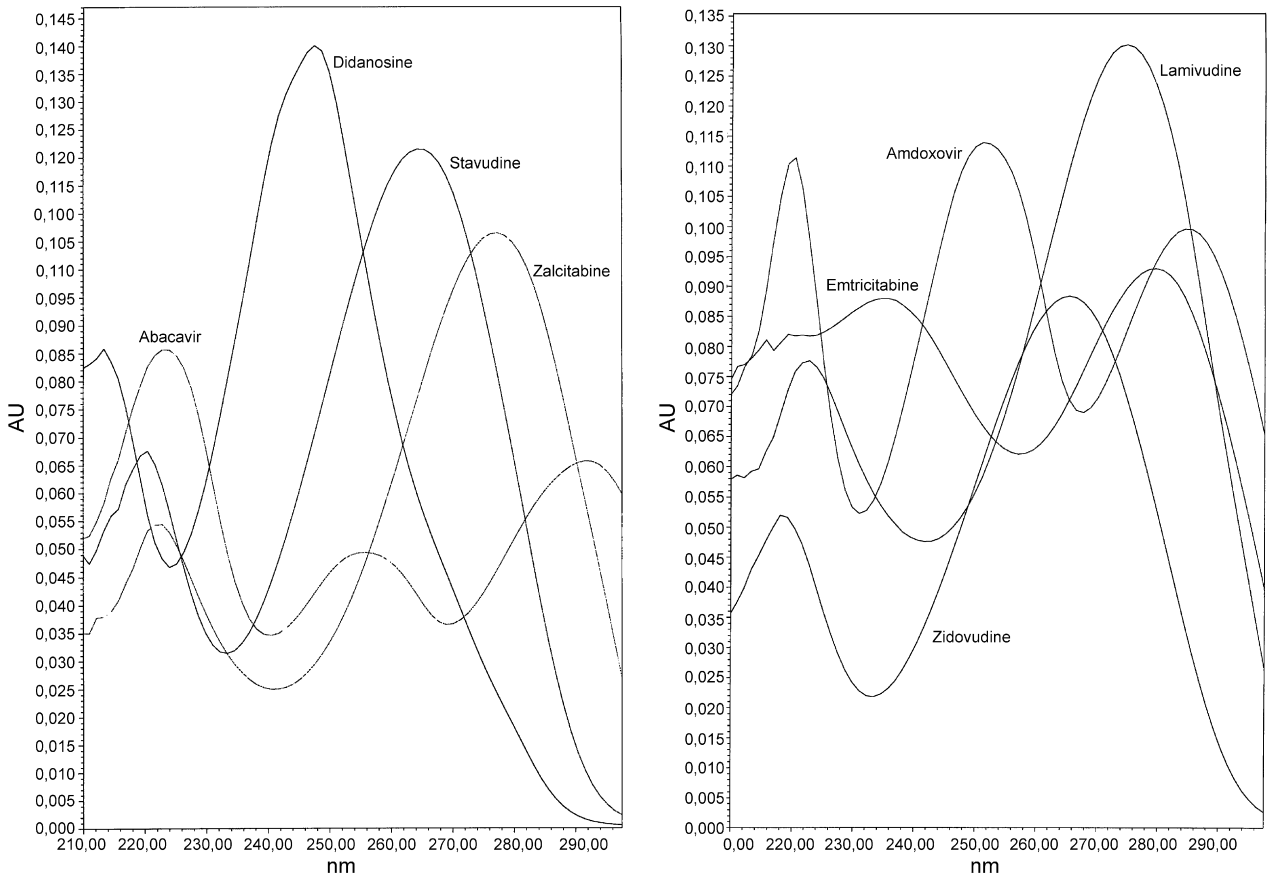

Fig. 3. UV spectra of IN family molecules.

3.4. Sample analysis

Several medicinal products (1 1 0) taken from the African and Asian markets were tested. Among these products, different forms were available, such as tablets, hard and soft capsules and oral suspensions. These were presented as mono-therapy (61), bi-therapy (24) and tri-therapy (25) products. The analysis involved determining the declared active ingredient content. Both HPLC methods made possible the verification of the absence of interference by excipients commonly used in tablet and capsule formulation. The developed methods were thus specific for ARV and excipients of tested products.

3.5. Solution stability

A stability study was specifically performed on didanosine. The molecule degrades by 30% in 6 h in acidic solutions (mobile phase) giving hypoxanthine as impurity. However, didanosine remains stable in aqueous solutions and in the solvent mixture (water 95%–methanol 5%). For this reason, all method 1 standard solutions were prepared in solvent

Table 4
Statistical study of method 1 for IN family

	Theoretical value	Results	Conclusion
LINEARITY			
Slope	/	6824 to 26806	
Y-intercept	/	−8936 to 8210	
Correlation coefficient	/	0.996 to 1.000	
Variances homogeneity (Cochran)	C (0.05; 6; 2) = 0.6161	0.5664 to 0.6085	Non significant
Y-intercept comparison with 0 (Student)	t (0.05; 16) = 2.1199	0.4515 to 1.1239	Non significant
Significant slope existence (Fisher)	F (0.001; 1; 16) = 16.1202	1848.9 to 16455.3	Highly significant
Validity of calibration curve (Fisher)	F (0.05; 4; 12) = 3.2592	0.1299 to 0.4214	Non significant
PRECISION			
Variances homogeneity (Cochran)	C (0.05; 3; 4) = 0.7457	0.4373 to 0.6114	Non significant
Repeatability RSD	/	0.41% to 0.84%	
Intermediate precision RSD	/	0.72% to 1.17% except didanosine = 5.7%	

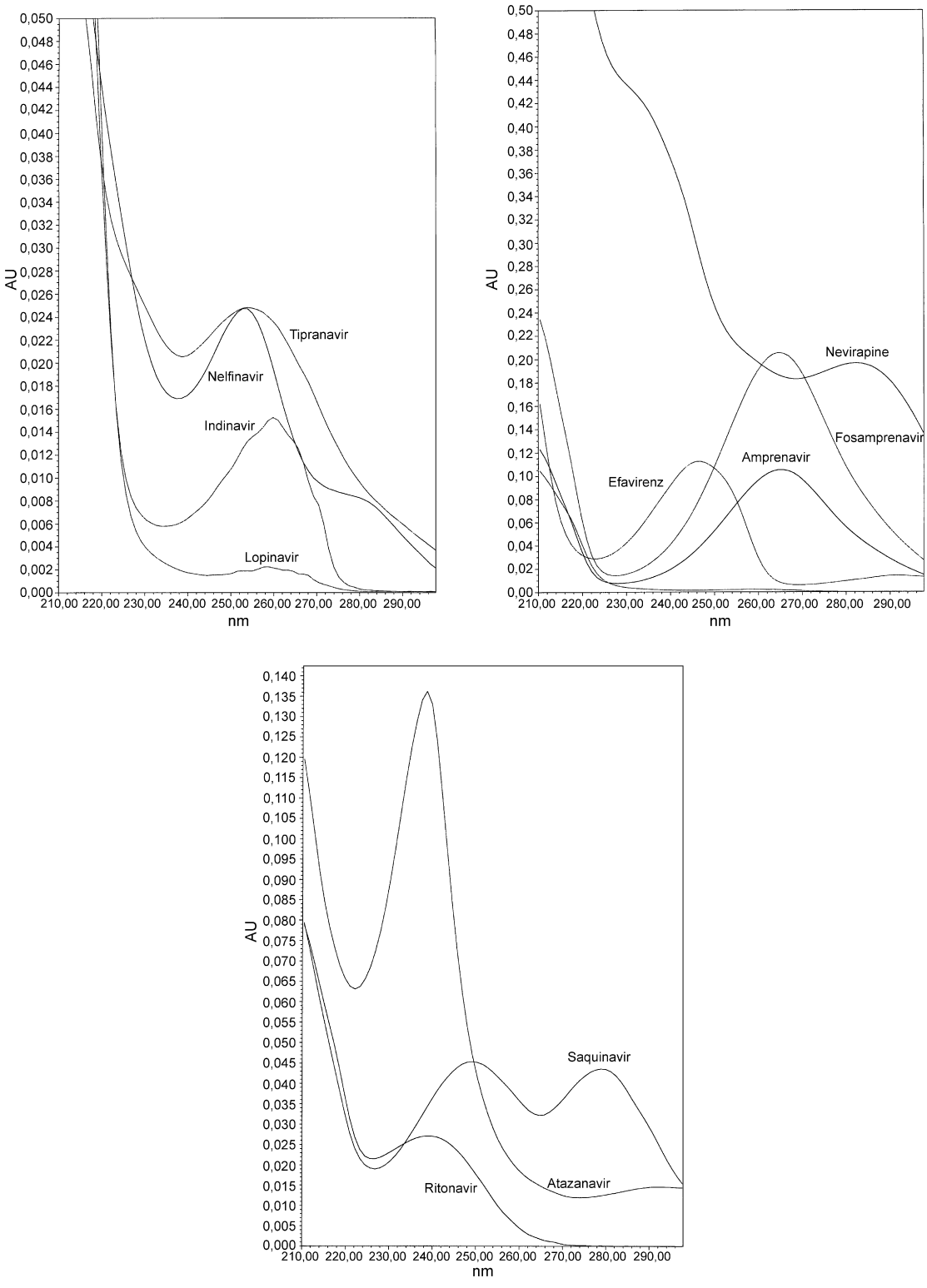

Fig. 4. UV spectra of INN and IP family molecules.

mixture (water 95%–methanol 5%). Didanosine, a fragile molecule in solution and unstable in acidic medium, must be injected within 4 h after being set in the water solution and diluted in the solvent mixture (water 95%–methanol 5%). Stability of others molecules in solution was shown over at least 48 h.

4. Discussion

The two developed HPLC methods allowed separation, identification and quantification of 19 antiretroviral agents. HPLC columns were reversed phase, and detection was done in the ultraviolet region at 270 nm and 260 nm. Mobile phases were

Table 5
Statistical study of method 2 for INN and IP families

	Theoretical value	Results	Conclusion
LINEARITY			
Slope	/	726 to 27576	
Y-intercept	/	−5141 to −518	
Correlation coefficient	/	0.999 to 1.000	
Variances homogeneity (Cochran)	C (0.05; 6; 2) = 0.6161	0.4193 to 0.5324	Non significant
Y-intercept comparison with 0 (Student)	t (0.05; 16) = 2.119905	0.1829 to 1.8682	Non significant
Significant slope existence (Fisher)	F (0.001; 1; 16) = 16.1202	8271.4 to 106203.5	Highly significant
Validity of calibration curve (Fisher)	F (0.05; 4; 12) = 3.2592	0.0166 to 1.7110	Non significant
PRECISION			
Variances homogeneity (Cochran)	C (0.05; 3;6) = 0.4160	0.4039 to 0.6706	Non significant
Repeatability RSD	/	0.30 to 0.79% except lopinavir = 1.97%	
Intermediate precision RSD	/	0.32% to 0.85% except lopinavir = 1.97%	

binary with a buffer gradient (pH 4.0 for method 1 and pH 5.65 for method 2) and an organic solvent (methanol for method 1 and acetonitrile for method 2). Several columns were tested (YMC-pack ODS-AQ, Stability RP18, YMC-pack ODS-AM and Symmetry C18) and gave chromatographic profiles similar to those obtained with the columns used for the validations. It was possible to transpose method 1 on a LC/MS system in order to obtain additional structural information [12,13]. Method 2 transposition was not initiated yet.

A UV mono wavelength detector can be used. The choice of detection wavelengths was made after studying absorption spectra of all the molecules. These maxima ranged between 240 nm and 290 nm; 270 nm was chosen for method 1 and 260 nm for method 2. Lopinavir had a maximum absorption at 260 nm, but its low signal brought coefficients of variation of repeatability and intermediate reliability slightly greater than the other compounds but always less than 2%. To confirm wavelength choice, the determination of several selected medicinal products was carried out with the presented method's wavelength and with that of the maximum of absorption of the molecule to be proportioned. These tests showed exactly the same results.

The solubility of all compounds was studied during the preparation of standards and sample solutions. The dead volume (1.5 min) of each method was determined by the injection of a non retained compound. The 19 studied molecules were suitably chromatographied. A compound from the IN family was withdrawn from the study: tenofovir disoproxil fumarate [6]. This compound appeared in dead volume with method 1. However, under our experimental conditions, the peak shape was symmetrical and did not show interference. Its UV spectrum was determined and its purity was calculated by the software. The validation of the method for this molecule's analysis was not scientifically possible, but tenofovir could be identified in a product if no other compound is detected in the dead volume.

The solutions were stable at 48 h minimum in their dilution solvent. Only didanosine presented rapid degradation in an acidic medium. It was, therefore, necessary to dissolve this compound in water, dilute it in the water 95%–methanol 5% mixture and then inject it within 4 h after preparing the sample.

The analysis lasted 60 min for method 1 and 50 min for method 2, but the separation of all the compounds was carried out in 45 min. Compounds were correctly separated and peak resolution was always greater or equal to 1.5. A series of three IN molecules was eluted in less than 1 min (between 23.4 and 24.4 min). The peak resolution was however correct because the resolution of the two nearest compound (didanosine and stavudine) was sufficient (according to the European pharmacopeia 5th edition). Moreover, the association of these two molecules does not exist in any commercial trade.

Method validation was based on a statistical study of linearity and precision. The calibration range selected was rather broad, from 10 μg/ml to 100 μg/ml, making possible in one single injection the analysis of products containing several active ingredients having wide variations in their quantities (example: lamivudine 150 mg, stavudine 30 mg). Method precision was checked with the average concentration of 50 μg/ml.

Analysis of 110 medicinal products issued from the African and Asian market allowed the specificity of the method to be checked. The absence of interference with the excipients of the analyzed formulations was emphasized, and the purity of the chromatographic peaks was shown. The controlled drugs were in the form of hard and soft capsules and tablets. These two methods demonstrated a tri-therapy medicinal product falsification: presence of the first ARV molecule, substitution of the second ARV molecule by another ARV compound and replacement of the third ARV compound by an inactive molecule. An analysis by LC/MS was able to determine the nature of the falsified molecule.

5. Conclusion

A research in scientific literature shows that several studies exist concerning antiretroviral compounds in biological samples. Some publications describe a simultaneous determination of several molecules [4–13]. For drug control, several HPLC techniques exist and allow the determination of one or two molecules. The emergence of antiretroviral generic medicinal products and the search for counterfeits, falsifications or fake

products in this field led to the need to develop new comprehensive methods.

Two complementary HPLC methods were developed for this purpose: The first studied 8 antiretroviral molecules of the IN class and the second studied 11 antiretroviral molecules of INN and IP classes. These two HPLC methods enabled the separation, identification and quantification of 19 molecules among the 26 compounds which are currently indexed. Of the seven remaining molecules, five are under development [2], one is not analyzable with any of the two methods (Tenofovir), three were not obtained (elvucitabine, delavirdine and capravirine) and three were not indexed at the beginning of the study (alovudine, etravirine and calanolide). Nevertheless, analysing 19 molecules made it possible to cover the majority of the antiretroviral substances commonly found in the international market.

Concentrations of these molecules used in medicinal products range from 30 mg to 600 mg per tablet or capsule. Analysing more than 110 products made it possible to check the absence of interference by excipients used for the formulation. The linearity established between 10 μg/ml and 100 μg/ml was checked for all the molecules as well as the analysis precision. The use of a photodiode array detector allowed an identification of the chromatographic peaks by their UV spectrum and the verification of their purity. Creating a library of raw material antiretroviral molecules in the laboratory provided a rapid answer to the whole of these qualitative and quantitative analyses.

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