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Validation of high-performance liquid chromatography methods for determination of zidovudine, stavudine, lamivudine and indinavir in human plasma

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Resumen

Objetivo: Fueron descritos y validados métodos analíticos simples para la determinación de zidovudina (AZT), estavudina (d4T), lamivudina (3TC), e indinavir (INV) en plasma humano por cromatografía líquida de alta resolución (HPLC) con detección UV.

Método: Se aplicó la extracción en fase sólida para la preparación de las muestras previo al análisis. La corrida cromatográfica se realizó en una columna analítica C-18 y el tiempo de retención se movió en un rango de 6,8-11,9 min para d4T, 7,5-9,0 para 3TC y 11,2-11,9 para AZT e INV. Se validaron 4 métodos en cuanto a especificidad, precisión y exactitud entre días y en el día, así como recobrado y estabilidad.

Resultados: Los rangos de concentraciones de las curvas analíticas eran de 10-1600 ng/ml para d4T, 50-3200 ng/ml para 3TC, 0,05-5,0 µg/ml para AZT y 0,1-10,0 µg/ml para INV. Se demostró la estabilidad del analito durante el procesamiento de las muestras y el almacenamiento. Para las 4 formulaciones los resultados del por ciento de recobrado fue superior al 89%.

Conclusiones: Estos métodos demostraron ser simples, exactos, precisos y son los utilizados actualmente en nuestro laboratorio para el análisis cuantitativo de productos antirretrovirales en plasma, así como para posteriores estudios de farmacocinética y bioequivalencia.

Palabras clave: Líquido cromatográfico. Extracción en fase sólida. Zidovudina. Estavudina. Lamivudina. Indinavir. Validación. HPLC.

Summary

Objective: Simple methods for the determination of zidovudine (AZT), stavudine (d4T), lamivudine (3TC) and indinavir (INV) in human plasma by reversed-phase liquid chromatography (HPLC) with UV detection were described and validated.

Method: Solid-liquid extraction procedures were applied to the samples prior to analysis. Chromatography was performed on a C-18 analytical columns and the retention time ranged from 6.8 to 8.0 min for stavudine, 7.5 to 9.0 min for lamivudine, 11.2 to 11.9 min for zidovudine and indinavir. Four methods were validated for specificity, inter-and intra-assay precision and accuracy, absolute recovery and stability.

Results: Analytical curve ranged from 10-1600 ng/ml for stavudine, 50-3200 ng/ml for lamivudine, 0.05-5.0 µg/ml for zidovudine and 0.1-10.0 µg/ml for indinavir. Analytes stability during sampling processing and storage were established. Extraction recoveries are higher than 89% for all formulations.

Conclusions: These methods proved to be simple, accurate and precise, and are currently in use in our laboratory for the quantitative analysis of antiretrovirals products in plasma, and for further pharmacokinetics and bioequivalence studies.

Key words: Liquid chromatography. Solid-phase extraction. Zidovudine. Stavudine. Lamivudine. Indinavir. Validation. HPLC.

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INTRODUCTION

Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples play a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. It is essential to employ well characterized and fully validated analytical methods to yield reliable results which can be successfully interpreted¹.

Combination therapy with three or more highly active antiretroviral agents is advocated for the treatment of HIV infection. Recommended first-line regimens include the use of two nucleoside reverse transcriptase inhibitors (NRTI) with either one or two protease inhibitors (PI)². Among these combination regimens AZT-3TC-INV or d4T-3TC-INV are very useful in the treatment with antiretroviral agents in our country and all over the world³⁻¹⁰.

Methods for analysis of the approved anti-HIV agents in blood serum or plasma have been developed to support the evaluation of clinical efficacy. These methods are the basis for the pharmacokinetic information available for each drug¹¹⁻³³.

This paper describes four validated methods combining solid-liquid extraction, reversed-phase liquid chromatography and UV detection to perform the selective determination of AZT, d4T, 3TC and INV.

METHOD

Chemicals

All used chemicals were HPLC-grade, from Merck (Darmstadt, Germany). The Marianao Blood Bank, Havana city, Cuba, supplied drug-free human plasma. Solid-phase extraction cartridges (C18 LiChrolut, 1 mL, 100 mg) were purchased from Merck (Darmstadt, Germany). Standards of zidovudine, stavudine, lamivudine and indinavir were obtained from Novatec Laboratory, Cuba.

Instrumentation

The EuroChrom 2000 chromatography manager software was used to control the HPLC system which consists of a K-1001 HPLC pump, a K-2600 UV variable detector, and the automatic injector "Basic Marathon", all from Knauer (Berlin, Germany).

The used stationary phase analytical run was C18 packed in a (250 x 4 mm) with 5 µm particle size column from Merck (Darmstadt, Germany). All analytical runs were preceded by a Security guard column LiChro-CART® 4-4, LiChrospher® 100 RP-18 (5 µm) from Merck (Darmstadt, Germany).

Chromatographic conditions

All chromatographic experiments were carried out in the isocratic mode at room temperature. The mobile phase was vacuum degassed before use. The mobile phase consisted of a mixture of acetonitrile and water (9:91, v/v) at a flow-rate of 0.7 ml/min for d4T and 3TC, methanol and water (20:80, v/v) at a flow-rate of 1.2 ml/min for AZT and buffer phosphate (pH 5.7, adjusted

with Na₂HPO₄) and acetonitrile (60:40, v/v) with 0.2% triethylamine at a flow-rate of 1.3 ml/min for INV. Aliquots of sample and standard solutions (50 µl) were injected onto the analytical column with the autosampler. The UV detector was used for monitor the drugs at wave lengths of 271 nm (d4T and 3TC), 267 nm (AZT) and 210 nm (INV).

Samples from the indinavir study were analyzed by a modified version of the Foisy ML assay³². The modification consisted in the addition of 0.2% triethylamine as ion-paired to ensure an appropriate retention time for INV to differentiate it from other potential interference from plasma peak.

Preparation of standard stock solutions

The powdered drugs were weighed and dissolved in bidistilled water at room temperature to obtain a stock solution of 1.0 mg/ml. Serial dilutions of the stock solutions were appropriately diluted to lower concentration for spiking the calibration standards. Calibration standards covering the concentration range between 10 to 1,600 ng/ml (10, 25, 50, 100, 400, 800, and 1,600 ng/ml) for d4T, 50 to 3,200 ng/ml (50, 100, 200, 400, 800, 1,600, and 3,200 ng/ml) for 3TC; 0.05 to 5.0 µg/ml (0.05, 0.1, 0.5, 1.0, 1.5, 2.5, and 5.0 µg/ml) for AZT and 0.1 to 10.0 µg/ml (0.1, 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 µg/ml) for INV. The simulate samples were prepared by adding appropriate volumes of the diluted solutions to drug-free human plasma. Aliquots of calibration standard, and samples were pipetted into screw-capped culture tubes, and keep frozen at -20 °C until required for analysis.

Sample preparation procedure

From the calibration standard solutions were prepared, in blank plasma, several concentrations for determinate the recovery of each method. These solutions contained 100, 400 and 800 ng/ml for 3TC; 10, 100 and 1,600 ng/ml for d4T; 0.1, 1.0, and 2.5 µg/ml for AZT; and 0.5, 1.0 and 5.0 µg/ml for INV. Three injections of each concentration were performed. The procedure of extraction was applied for all plasma samples of all formulations. The solid-phase extraction cartridge was conditioned with 2 ml of methanol and 2 ml of bidistilled water, before 500 µl of plasma samples were loaded into the column and were allowed to pass through the bed with minimal suction followed by 500 µl of methanol. The eluent was evaporated to dryness under a nitrogen stream at 40 °C, the residue was reconstituted in the mobile phase (200 µl), and an aliquot (50 µl) was injected onto the HPLC system.

Validation of analytical methods

—*Specificity.* Specificity of the methods were demonstrated by extracting and analyzing spiked AZT, 3TC, d4T and INV from five different lots of normal human plasma.

—*Linearity.* Calibration plots for the analytes in plasma were prepared by spiking drug-free plasma with standard stock solutions to yield concentrations of 10 to 1,600 ng/ml (10, 25, 50, 100, 400, 800 and 1,600 ng/ml) for stavudine, 50 to 3,200 ng/ml (50, 100, 200, 400, 800, 1,600 and 3,200 ng/ml) for lamivudine, 0.05 to 5.0 µg/ml (0.05, 0.1, 0.5, 1.0, 1.5, 2.5 and 5.0 µg/ml) for zidovudine and 0.1 to 10.0 µg/ml (0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 µg/ml) for indinavir.

Five injections of each concentration were performed. Calibration curves were constructed using ratios of the observed analyte peak area versus concentration of analyte. Linear regression analysis of the data gave slope, intercept and correlation coefficient data which were then used to calculate analyte concentration in each sample.

—*Limit of detection (LOD) and limit of quantification (LOQ).* Plasma samples were spiked with decreasing concentrations of the analytes and analyzed. The LOD was defined as the concentration that yields a signal-to-noise ratio of 3. The LOQ was determined as the percentage deviation from the nominal concentration and were less than 20%³³.

—*Recovery.* The recoveries of each drug from plasma were determinate by comparing the peak area of each analyte after extraction with the respective non-extracted standard solutions at the same concentration.

—*Stability.* Stability of AZT, 3TC, d4T and INV in solution and in biological matrix was determined at two different temperatures (at 25 and 57 °C).

RESULTS

Validation data are shown in table I.

—*Chromatographic characteristics.* The figure 1 shows chromatograms, respectively, an extracted blank plasma sample (A), an extracted plasma containing AZT (B), an extracted plasma containing 3TC (C), an extracted plasma containing d4T (D), and an extracted plasma containing INV (E). Retention times ranged from 6.8 to 8.0 min for d4T, 7.5 to 9.0 min for 3TC, 11.2 to 11.9 min for AZT and INV (Table I).

—*Calibration curve.* The analysis of zidovudine, lamivudine, stavudine and indinavir in plasma showed excellent linearity ($r^2 = 0.9996$, $r^2 = 0.9975$, $r^2 = 0.9984$, $r^2 = 0.9834$) respectively, over the concentration ranges studies. Regression intercepts for the calibration curves were generally very small and were not statistically significant compared to zero. The intercepts and CV are shown in table I. The daily fluctuation of plasma standard curves was slight.

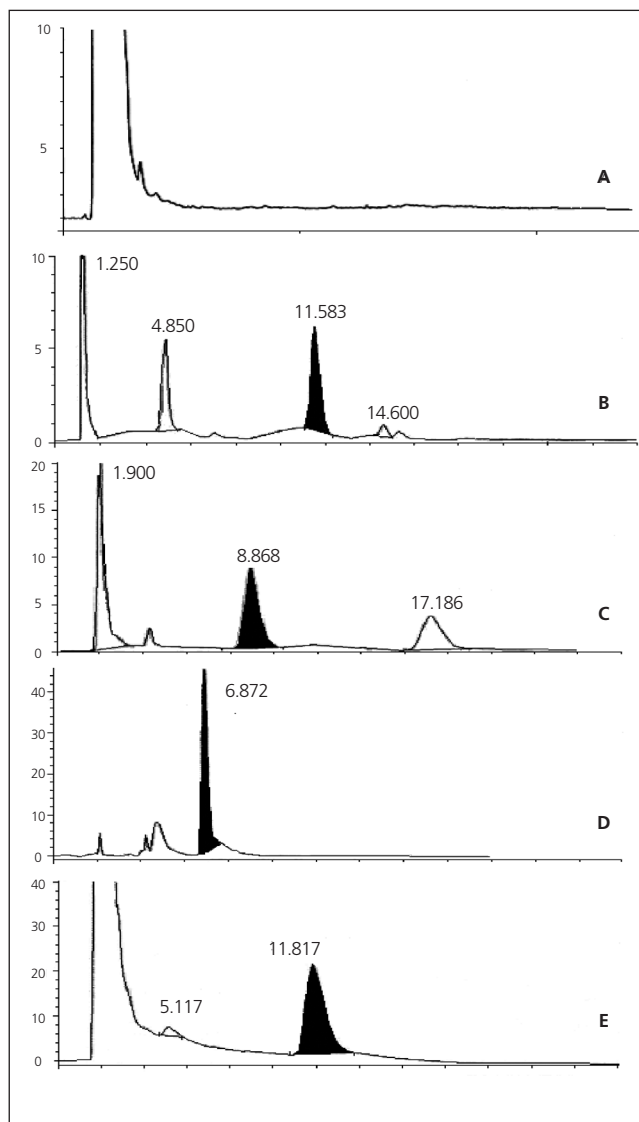


Fig. 1. Chromatograms showing: (A) Blank human plasma; (B) Sample extract zidovudine. (Concentration = 1.20 µg/ml); Column: C18, 250 x 4 mm, 5 µm; mobile phase: methanol-water (20:80, v/v); isocratic flow: 1.2 ml/min; (C) Sample extract lamivudine. (Concentration = 800 ng/ml). Column: C18, 250 x 4 mm, 5 µm; mobile phase: acetonitrile-water (9:91, v/v); isocratic flow: 0.7 ml/min; (D) Sample extract stavudine. (Concentration = 1600 ng/ml). Column: C18, 250 x 4 mm, 5 µm, mobile phase: acetonitrile-water (9:91, v/v); isocratic flow: 0.7 ml/min; (E) Sample extract indinavir. (Concentration = 2.50 µg/ml). Column: C18, 250 x 4 mm, 5 µm, mobile phase: buffer phosphate (pH 5.7, adjusted with Na₂HPO₄)-acetonitrile (60:40, v/v), 0.2% triethylamine; isocratic flow: 1.3 ml/min.

—*Accuracy, precision and recovery.* For the inter-assay precision, the CV, ranged from 0.40 to 12.38% for all the antiretroviral studies. The means accuracy ranged from 70 to 126.8%. For the intra-assay precision, the CV, ranged from 0.26 to 11.88% for all the antiretroviral studies. The means accuracy ranged from 6.0 to 150% (Table I). This highlights the methods sensitivity. These results indicated that these assays were reliable and reproducible.

Table I. Validation data

	Stavudine	Lamivudine	Zidovudine	Indinavir
t_R (min)	6,8-8,0	7,5-9,0	11,2-11,9	11,2-11,9
r (n = 5)	0,9992	0,9988	0,9998	0,9915
CV (%)	0,08	0,17	0,01	0,33
Intercept	-0,0245	0,8187	-0,0231	0,3231
r^2 (n = 5)	0,9984	0,9975	0,9996	0,9834
CV (%)	0,16	0,33	0,01	0,69
Inter-day (n = 3)	10-1600	50-3200	0,05-5,0	0,1-10,0
	(ng/ml)	(ng/ml)	(μ g/ml)	(μ g/ml)
Precision (%)	0,40-7,9	3,53-6,61	7,12-12,38	2,79-11,60
Accuracy (%)	94,0 - 126,8	98,0-105,0	70,0-103,0	84,9-107,2
Intra-day (n = 5)	10-1600	50-3200	0,05-5,0	0,1-10,0
	(ng /mL)	(ng /mL)	(μ g/mL)	(μ g/mL)
Precision (%)	0,45-11,88	3,30-9,33	0,26-8,35	1,79-9,46
Accuracy (%)	96,0-101,2	93,6-104,3	60,0-150,0	84,9-120,0
LOQ (n = 5)	10 ng/ml	50 ng/ml	0,05 μ g/ml	0,1 μ g/ml
CV (%)	11,9	3,30	13,30	13,60
Accuracy (%)	96,0	94,8	125,0	140,0
LOD (n = 5)	5 ng/ml	11.5 ng/ml	0.01 μ g/ml	0.05 μ g/ml
CV (%)	19,1	14,3	4,44	3,77
Absolute recovery (%)	91,0	95,9	90,0	89,3

t_R : retention time; r : regression slope; CV: coefficient of variation; r^2 : correlation coefficient; LOQ: limit of quantitation (< 20%); LOD: limit of detection.

The means absolute recovery of the stavudine, lamivudine, zidovudine and indinavir were 91, 95.9, 90 and 89.3% respectively.

—*Selectivity.* Blank human plasma samples showed no interfering endogenous substances eluting at the retention time of AZT, 3TC, d4T and INV (Figs. 1 A-E).

—*Limit of detection (LOD) and limit of quantitation (LOQ).* Each LOD and the LOQ with their CV respective are including in the table I. LOQ levels were included in

the calibration curves as the lowest concentration level. The lower LOQ for stavudine, lamivudine, zidovudine and indinavir had a CV between 3.30 to 13.60% and the mean accuracy was 94.82 to 140.0% of the target concentration in human plasma.

—*Stability.* No significant loss of AZT, 3TC, d4T, and INV were observed by heaten. We found that these anti-HIV drugs in plasma samples were stables during a heat deactivation of HIV at 57 °C for 40 min compared with fresh samples. This finding was confirmed by the fact that no difference were observed between peak heights of pure standards of AZT, 3TC, d4T, and INV prepared in water, whether or not they were heated to 57 °C for 40 min before being injected into the column.

DISCUSSION

The specific, sensitive and rapid methods have been validated for the quantitation of zidovudine, lamivudine, stavudine and indinavir in human plasma samples. The applicability of the assays for pharmacokinetic and bioequivalence researches in HIV-1 infected patients was possible. Thus, monitoring zidovudine, lamivudine, stavudine and indinavir pharmacokinetics may be imperative to ensure optimal drug efficacy and to control the tolerability profile during the initiation of treatment. In conclusion, validated assays for the quantitation determination of zidovudine, lamivudine, stavudine and indinavir in human plasma have been described. The HPLC methods should be useful for monitoring plasma drug concentrations and pharmacokinetic studies in HIV-infected patients and for any other studies were it is desirable to measure concentrations of these anti-HIV agents in biological samples.

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