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Journal of Chromatography B, 742 (2000) 173–183

JOURNAL OF CHROMATOGRAPHY B

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Simultaneous determination of lamivudine and zidovudine concentrations in human seminal plasma using high-performance liquid chromatography and tandem mass spectrometry

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Received 27 October 1999; received in revised form 28 February 2000; accepted 28 February 2000

Abstract

A HPLC–MS–MS method was developed and validated to measure lamivudine and zidovudine simultaneously in small volumes of human seminal plasma. Sample preparation was simple and rapid, requiring 25 μl of sample, the use of isotopically labeled lamivudine and zidovudine as internal standards and ultrafiltration through a molecular mass cut-off membrane. Lamivudine and its internal standard were separated from zidovudine and its internal standard with isocratic HPLC. Detection was carried out using tandem mass spectrometry. This validated method was used to analyze seminal samples obtained from six HIV-positive patients prescribed lamivudine and zidovudine.

Keywords: Lamivudine; Zidovudine

1. Introduction

Human immunodeficiency virus type-1 (HIV-1), the agent that causes acquired immunodeficiency syndrome (AIDS), is found in blood, spleen, lymph nodes, brain, saliva, cervicovaginal secretions and semen of infected male patients [1–3]. Evidence suggests that anatomically distinct populations of virus exist under different evolutionary selective pressure. For example, HIV-1 isolated from semen has genetic characteristics that differ from the HIV-1 isolated from blood [4]. This suggests semen may be a privileged, or sanctuary, site of viral growth, not affected by systemic antiviral therapy. Contact with semen is the major route for the sexual transmission of HIV-1 [5]. Virus from whole semen, seminal nonspermatozoal mononuclear cells and cell free seminal plasma has been shown to infect plasma blood mononuclear cells in culture [5].

Synthetic nucleoside analogs are commonly used to treat HIV-1 infection. Human intracellular kinases phosphorylate these synthetic compounds to form 5′-triphosphorylated analogs, which are inserted into

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00162-6

the viral DNA. This insertion blocks further elongation of viral DNA. For this reason, compounds in this class of drugs are commonly called nucleoside analog chain terminators. Lamivudine (2′-deoxy-3′-thiacytidine, 3TC) and zidovudine (3′-azido-3′deoxythymidine, AZT, ZDV) are two commonly used drugs of this class [6].

The use of zidovudine plus lamivudine combination therapy causes a decrease of HIV-1 in blood plasma [7]. Although early studies suggested antiviral drugs have no effect on the seminal compartment [8], recent studies have reported that viral load in seminal plasma decreases following lamivudine plus zidovudine therapy [9,10]. However, none of these studies measured the concentrations of antiviral drugs in the seminal compartment.

Quantitative high-performance liquid chromatography (HPLC)–UV assays to measure zidovudine or lamivudine in human plasma and urine have been well described in the literature [11–16]. Several immunoassays have also been developed to measure zidovudine, including a commercially available radioimmunoassay kit, an enzyme-linked immunosorbent assay (ELISA) method, and a fluorescence polarization immunoassay [17–19]. These immunoassays allow for a smaller sample size, have greater sensitivity, and require less analysis time per sample than the HPLC–UV techniques. The radioimmunoassay kit has been used to measure zidovudine in both blood and seminal plasma [20]. However, until the recent development of a HPLC–tandem mass spectrometry (MS–MS) method by Kenney et al. [21], quantification of lamivudine and zidovudine required separate analyses, using HPLC–UV for lamivudine quantification and an immunoassay for zidovudine analysis. The HPLC–MS–MS technique has been shown to be a highly specific and sensitive method for the simultaneous measurement of both zidovudine and lamivudine in blood plasma.

This paper describes a validated HPLC–MS–MS method to measure lamivudine and zidovudine concurrently in human seminal plasma using stable, isotopically labeled zidovudine and lamivudine as internal standards (Fig. 1). Analysis is simple and rapid, requiring addition of an ammonium acetate solution containing both the lamivudine and zidovudine internal standards to the seminal plasma and ultrafiltration of the resulting mixture through a molecular mass cut-off membrane. Lamivudine and its internal standard and zidovudine and its internal standard are rapidly resolved using isocratic HPLC. A TurboIon Spray® source with tandem mass spectrometry is used to detect lamivudine, with its internal standard, in the positive ion mode and zidovudine, with its internal standard, in the negative ion mode.

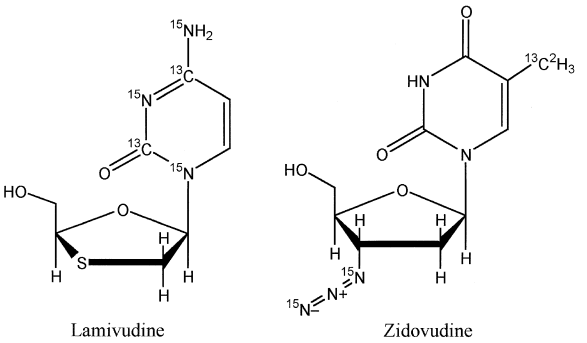

Fig. 1. Chemical structures of lamivudine and zidovudine internal standards, with isotopic labels shown in bold. The zidovudine internal standard is labeled at either azido nitrogen, but not both.

2. Experimental

2.1. Materials

Zidovudine, lamivudine, and their stable, isotopically labeled internal standards were prepared at Glaxo Wellcome (Research Triangle Park, NC, USA). The isotopes were five mass units greater than the parent compound (Fig. 1). The identity of the labeled zidovudine nitrogen is uncertain. Due to the synthetic steps involved, 50% of the terminal azido nitrogens are isotopically labeled and 50% of the internal azido nitrogens are isotopically labeled. Control bull semen was obtained without antibiotics and other additives from Select Sires (Plain City, OH, USA). Human semen was collected, with informed consent, from HIV-1 negative, antiretroviral naive volunteers at UNC Hospitals (Chapel Hill, NC, USA). Clinical seminal samples were obtained, with informed consent, from volunteer HIV-1 positive patients prescribed lamivudine and zidovudine as antiviral therapy. HPLC-grade water and acetonitrile were obtained from EM Science (Cincinnati, OH,

USA). Analytical grade ammonium acetate and acetic acid were obtained from Mallinckrodt (St. Louis, MO, USA). Disposable CentriFree ultrafiltration units with a molecular mass cut-off of 30 000, were purchased from Amicon (Beverly, MA, USA).

2.2. Instrumentation

The HPLC system consisted of a dual-pumping scheme controlled by a Rheodyne switching valve (Coati, CA, USA). A Waters 616 pump (Milford, MA, USA) was used to elute compounds of interest from the analytical column. Solvent from a secondary pump (Varian 9012, Palo Alto, CA, USA) bypassed the column and flowed to the mass spectrometer during the first 1.9 min of each analytical run, while the switching valve diverted flow from the primary pump to waste. An API 300 triple quadrupole mass spectrometer (PE Sciex, Norwalk, CT, USA) equipped with a TurboIon Spray® interface was used to determine levels of lamivudine, zidovudine and their internal standards.

2.3. Chromatographic conditions

The mobile phase of acetonitrile–water (15:85, v/v) was used to elute the samples through an Aquasil (C_{18} 5 μm, 150×2.1 mm) analytical column (Keystone Scientific, Bellafonte, PA, USA) at a flow-rate of 0.3 ml/min. The secondary mobile phase bypassed the column, consisted of acetonitrile–water (55:45, v/v), and had a flow-rate of 0.3 ml/min. A needle wash of acetonitrile–water (50:50, v/v) preceded each injection. Typical injection volume was 25 μl. Separation was carried out at room temperature.

2.4. Mass spectrometric conditions

The mass spectrometer was connected to the HPLC system by a TurboIon Spray® interface (PE Sciex). Nitrogen was used as both curtain and nebulizer gases. User controlled voltages, gas pressures and source temperature were optimized for the detection of the parent and product ions of lamivudine and zidovudine (Table 1). Lamivudine was monitored in the positive ion mode and zidovudine in the negative ion mode. The first quadrupole, Q1, was optimized for the maximum transmission of the protonated molecule of lamivudine (m/z 230) and its internal standard (m/z 235) and the deprotonated molecule of zidovudine (m/z 266) and its internal standard (m/z 271). Nitrogen was also used as the collision gas for collision activated dissociation (CAD). Product ions were monitored for lamivudine (230→112), its internal standard (235→117), zidovudine (266→223), and its internal standard (271→227). Both Q1 and Q3 were operated at unit mass resolution. The dwell time for each transition was 500 ms with a 5 ms pause between scans.

2.5. Data acquisition and analysis

Data acquisition was performed using multiple reaction monitoring of lamivudine along with its internal standard and zidovudine along with its internal standard. For the first 4 min of the run, the

Table 1
Operating conditions for monitoring lamivudine and zidovudine (V=volts)

Value (units)	Lamivudine, Positive ion mode	Zidovudine, Negative ion mode	Value (Units)	Lamivudine, Positive ion mode	Zidovudine, Negative ion mode
Nebulizer gas	10	10	Inter-quadrupole lens (V)	−7.5	9.0
Curtain gas	6	8	First resolving quadrupole (V)	−7.5	9.0
CAD gas	3	4	Inter-quadrupole lens (V)	−21.0	20.0
Source (V)	5000	−5000	Collision cell quadrupole (V)	−22.0	21.0
Temperature (°C)	450	450	Inter-quadrupole lens (V)	−40.0	39.0
Orifice (V)	24	−34.7	Last resolving quadrupole (V)	−24.0	23.0
Focusing ring (V)	375	−349.8	Deflector (V)	−400	400
Entrance quadrupole (V)	−6.5	8.0	Electron multiplier (V)	2250	2200

transitions of lamivudine and its internal standard were monitored in the positive ion mode. After 4 min, the transitions for zidovudine and its internal standard were monitored in the negative ion mode. Automated data acquisition and analysis were performed using the PE Sciex SampleControl application.

Post-acquisition quantitative analyses were preformed using MacQuan software (PE Sciex). Unknown sample concentrations of lamivudine were calculated from the equation $y=mx+b$, as determined by the weighted $(1/x^2)$ linear least-squares regression of the calibration line constructed from the peak area ratios of lamivudine to its internal standard versus lamivudine concentration. Unknown sample concentrations of zidovudine were calculated in a similar fashion using the weighted regression of the calibration line constructed from the peak area ratios of zidovudine and its internal standard.

2.6. Semen pretreatment

All semen samples were allowed to liquefy for 1 h at room temperature. All bull semen was pooled into one lot, the antiretroviral naive human semen into another. Each human clinical sample was processed separately. To collect seminal plasma, all semen was centrifuged for 30 min at 1000 *g*. The seminal plasma was collected and heated in an oven for 3 h at 58°C to inactivate HIV-1 [22]. Seminal plasma was kept frozen at −5°C until needed.

2.7. Preparation of stock solutions

Two independent initial stock solutions containing 100 ng/μl of both zidovudine and lamivudine in water were prepared. Aliquots of the stock solutions were diluted with water to form two intermediate stock solutions of 10 ng/μl and 1 ng/μl. These initial and intermediate stock solutions were stored at −5°C. One set of initial and intermediate stock solutions was used for the preparation of calibration standards; the other set for the preparation of quality control and dilution control samples.

2.8. Preparation of standards

Calibration standards were prepared in bull seminal plasma and used to construct calibration curves. First, calibration standards at concentrations of 5 and 10 ng/ml were prepared by dilution of the 1 ng/μl intermediate stock solution with bull seminal plasma. The 50 and 100 ng/ml calibration standards were prepared by dilution of the 10 ng/μl intermediate stock solution with bull seminal plasma. Finally, 500, 1000, 2500 and 5000 ng/ml calibration standards were prepared by dilution of the 100 ng/μl stock solution with bull seminal plasma. The calibration standards were stored at −5°C.

2.9. Preparation of quality controls and dilution controls

Quality control and dilution control samples were prepared in human seminal plasma. Quality control samples were used to measure the accuracy and precision of the assay throughout the entire procedure. Dilution control samples were used to ensure dilution of clinical samples with ammonium acetate during extraction did not affect accuracy or precision.

The quality control samples contained solutions of 5, 50, 500, 2500 and 5000 ng/ml zidovudine and lamivudine. The 5 and 5000 ng/ml quality controls were used to establish the accuracy and precision at the lower and upper quantitation limits of the standard curves. The quality control sample at 5 ng/ml was prepared by dilution of the 1 ng/μl intermediate stock solution with human seminal plasma.. The 50 ng/ml quality control sample was prepared by dilution of the 10 ng/μl intermediate stock solution with human seminal plasma. The 500, 2500 and 5000 ng/ml quality control solutions were prepared by dilution of the 100 ng/μl stock solution with human seminal plasma.

Dilution control samples contained solutions of 100, 500, 5000 and 10 000 ng/ml zidovudine and lamivudine. All dilution control samples were prepared by dilution of the 100 ng/μl stock solution with human seminal plasma. These quality control and dilution control samples were stored at −5°C.

2.10. Preparation of internal standard solution

A stock solution containing 100 ng/μl of the isotopically labeled zidovudine and lamivudine internal standards in water was prepared. This stock

solution was stored refrigerated. The final, working solution of 500 ng/ml was prepared by diluting the stock solution with a 25 m*M* ammonium acetate solution that had been adjusted to a pH of 5.0 with acetic acid.

2.11. Extraction procedure

Aliquots of each calibration standard, quality control sample, dilution control sample and clinical sample were pipetted into separate CentriFree tubes. The sample size for calibration standards, undiluted clinical samples and quality control samples was 75 μl. The sample size was 25 μl for dilution control samples and diluted clinical samples. An additional 50 μl of 25 m*M* ammonium acetate pH adjusted to 5.0 with acetic acid was added to the dilution control samples and the clinical samples. All samples were spiked with 75 μl of the internal standard solution, capped, vortexed, and allowed to equilibrate at room temperature for 30 min. The tubes were then centrifuged for 30 min in a fixed angle centrifuge at 3000 *g*. Finally, the filtrate from each tube was transferred to individual HPLC injection vials and introduced into the HPLC system by an automatic sample injector.

2.12. Assay validation

Four consecutive analytical runs were used to determine the appropriate calibration model, the calibration range of the method, and the accuracy and precision of the assay. Each analytical run contained duplicate standard curves, two double blank samples, six replicates of each quality control concentration, and six replicates of each dilution control concentration.

Specificity of the method for lamivudine, zidovudine, and their respective internal standards was tested using both bull and drug free human semen. These double blank samples were analyzed without addition of stock solution or internal standard solution to check for interfering endogenous co-eluting peaks.

The calibration model describes the mathematical relationship between the peak area under the curve ratio of analyte:internal standard and the analyte concentration. A linear regression model ($y=mx+b$) was assumed prior to validation. To determine the appropriate weighting scheme (unweighted, $1/x$, $1/x^2$), six replicates of each calibration control were analyzed. Three calibration curves were constructed using results from these replicates and the three different weighting schemes. The concentration of each standard was determined. The difference between nominal and back calculated concentration of each standard was then determined. By adding the absolute values of these differences under the three different weighting schemes, three different total errors were calculated. The weighting that gave the lowest total error was chosen and used throughout the remainder of this validation procedure and during subsequent analytical runs.

Accuracy describes the agreement between measured and nominal concentrations. Quality control and dilution control samples analyzed during the four consecutive analyses were used to calculate the accuracy at each quality and dilution control concentration. In all, 24 replicates of each quality control and dilution control concentration were analyzed (six replicates per day for 4 days). The overall mean of each quality control and dilution control concentration was determined and used to calculate the percent bias (% bias) for each concentration. For validation, the % bias must be less than or equal to 15% at all quality control and dilution control concentrations.

Precision is the agreement among replicate measurements. Within-day precision is the variation of the each of the six daily replicates for each concentration. Between-day precision is the variation across the 4 days for each concentration. The within- and the between-day precisions of the method were calculated using the four analytical runs described above. One-way analysis of variance (ANOVA) testing was used to give estimates of within- and between-day precision for each concentration. These were reported as relative standard deviations (RSDs) for each concentration. For method validation, within- and between-day RSDs must be less than or equal to 15% at all quality control and dilution control concentrations.

The limits of quantitation (LOQs) are the highest and lowest concentrations of analyte that can be measured with acceptable accuracy and precision (% bias and RSD less than or equal to 15%). The upper

and lower limits of quantitation were determined by the quality control samples analyzed during the four analytical runs. The lower limit of quantitation was defined as the least concentrated quality control with acceptable accuracy and precision. The upper limit of quantitation was defined as the most concentrated quality control.

Dilution controls were analyzed to assess the linearity of dilution. Acceptable accuracy and precision (% bias and RSD less than or equal to 15%) at each dilution control concentration is required to ensure linearity of dilution.

2.13. Stability testing

One analytical run was conducted to ensure analyte stability in matrix. Six aliquots of a 2500 ng/ml lamivudine and zidovudine spiked human sample were prepared and stored for nine months, as described above. These were thawed and analyzed in tandem with six aliquots of a freshly prepared 2500 ng/ml lamivudine and zidovudine spiked human sample. In order to demonstrate long term stability, separate two one-sided *t*-tests (TOSTs) were performed at the 95% level of confidence, one TOST for lamivudine and one TOST for zidovudine. The TOST compares the mean difference between fresh and thawed samples by testing their equivalence, not equality. The procedure finds a 90% confidence interval (CI) for the true mean difference. The groups are equivalent if the whole CI lies within suitable tolerance limits, which in this case are taken to be within 15% of the reference (or freshly prepared) mean. Such a procedure is known to test for equivalence at the 5% level. The method was described by Timm et al. [23].

2.14. Clinical samples

Clinical samples were obtained from six individuals using lamivudine plus zidovudine as combination antiviral therapy. Two aliquots of these samples were processed and analyzed as described above. To again show linearity of dilution, one aliquot was diluted with ammonium acetate dilution and the other was not.

3. Results and discussion

3.1. HPLC–MS–MS assay validation

A HPLC–MS–MS method for the quantitation of lamivudine and zidovudine in human seminal plasma has been developed. Through rigorous validation tests, this method was shown to have acceptable specificity, precision and accuracy. The method described here is based upon previous work performed by Kenney et al. [21], who described a method to quantitate lamivudine and zidovudine in human blood plasma using tandem mass spectrometry. Initial assay validation showed transitions monitored by Kenney et al. on a PE Sciex API 3 tandem mass spectrometer are not ideal for analysis on a PE API Sciex 300 tandem mass spectrometer, due to differences in sources and quadrupole technology. While using a PE Sciex API 300, the transitions of 230→112, 235→117, 266→223 and 271→227 (lamivudine, lamivudine internal standard, zidovudine and zidovudine internal standard, respectively) were more intense than the previously reported transitions. As validation proceeded, it became evident that the use of human blood plasma as the matrix for calibration standards was inappropriate. Because large volumes of antiretroviral naïve human seminal plasma are difficult to obtain, bull seminal plasma was chosen as a surrogate matrix. As described below, further validation testing, using bull semen as the matrix for calibration standards and human semen as the matrix for quality and dilution controls, showed this substitution to be acceptable.

Assay validation requires choosing an appropriate calibration model, determination of the calibration range, evidence of specificity, and a high degree of precision and accuracy. Four consecutive analytical runs were used for validation. Each run contained duplicate analysis of each calibration standard, six replicates of each quality control, and six replicates of each dilution control concentration. Thus, 24 replicates (4 days×6 replicates) of each quality control and dilution control concentration were analyzed. The daily means and standard deviations of these replicate lamivudine and zidovudine concentrations are shown in Tables 2 and 3.

Specificity of this method was examined using drug free human and bull seminal plasma without the

Table 2
Statistical summary of four analytical assays for lamivudine concentration in replicate quality and dilution controls (SD=standard deviation)

Assay	Concentration (ng/ml)	Mean±SD	Assay	Concentration (ng/ml)	Mean±SD
1	*Quality control*		3	*Quality control*	
	5	5.6±0.5		5	5.0±0.8
	50	51.5±2.4		50	52.5±2.2
	500	536.2±29.3		500	545.7±25.8
	2500	2510±58.9		2500	2631±131.0
	5000	4879±172.4		5000	5424±313.7
	Dilution control			*Dilution control*	
	100	105.4±9.8		100	94.2±6.5
	500	509.2±30.0		500	518.6±46.0
	5000	5101±260.4		5000	4937±177.5
	10 000	10 970±427.7		10 000	5424±313.7
2	*Quality control*		4	*Quality control*	
	5	4.8±0.5		5	5.3±1.0
	50	50.4±2.8		50	55.6±4.8
	500	508.7±31.0		500	517.8±37.7
	2500	2381±221.1		2500	2358±137.5
	5000	4866±157.5		5000	5104±364.3
	Dilution control			*Dilution control*	
	100	97.9±6.0		100	90.2±4.1
	500	520.0±38.6		500	496.7±107.8
	5000	5620±226.5		5000	5566±241.6
	10 000	10 220±355.0		10 000	11 630±265.9

addition of any stock or internal standard solutions. No interfering endogenous materials were found in either the bull or human double blank samples. Typical double blank chromatograms are shown in Fig. 2.

To determine the appropriate calibration model (unweighted, $1/x$, $1/x^2$) to describe the peak area ratio of analyte:internal standard and nominal analyte concentration, calibration curves were created using the bull derived calibration standards using different weighting factors. Using a $1/x^2$ weighting factor, the smallest total absolute error and the largest correlation coefficient (r^2) were obtained. r^2, determined with a $1/x^2$ weighting factor, was greater than 0.995 for both lamivudine and zidovudine. The weighted calibration curve created for lamivudine was linear with a slope of 0.002 and *y*-intercept of 0.000. The linear weighted calibration curve for zidovudine had a slope of 0.002 and a *y*-intercept of 0.001. Interpolation from linear calibration curves using the $1/x^2$ weighting model was used throughout the remainder of the assay validation and during quantitation of clinical samples.

The overall accuracy, using all 24 replicates, was determined for each quality control and dilution control lamivudine and zidovudine concentration (Tables 4 and 5). All concentrations fell within the acceptable accuracy range (% bias <15%). The greatest % bias within the quality control set was 5.4% for lamivudine at 500 ng/ml and 8.2% for zidovudine at 5 ng/ml and 50 ng/ml. The greatest % bias within the dilution control set was 6.7% for lamivudine at 10 000 ng/ml and 7.5% for zidovudine at 5000 ng/ml.

Within-day precision is the degree of variation between the six daily replicates and between-day precision is the degree of variation between the four analytical runs. One-way ANOVA was used to determine precision at each concentration (Tables 4 and 5). All concentrations fell within the acceptable precision range (RSD<15%). The largest error for lamivudine in the quality control was at 5 ng/ml with a within-day RSD of 14.3% and a between-day RSD of 5.0%. The largest error for zidovudine in the quality control set also occurred at 5 ng/ml (9.9% within-day and 7.6% between-day). Lamivudine in

Table 3
Statistical summary of four analytical assays for zidovudine concentration in replicate quality and dilution controls (SD=standard deviation)

Assay	Concentration (ng/ml)	Mean±SD	Assay	Concentration (ng/ml)	Mean±SD
1	*Quality control*		3	*Quality control*	
	5	5.6±0.7		5	5.5±0.3
	50	53.9±2.3		50	52.8±1.8
	500	537.0±25.5		500	546.2±35.1
	2500	2649±66.8		2500	2682±222.0
	5000	5475±135.7		5000	5326±137.7
	Dilution control			*Dilution control*	
	100	98.2±3.9		100	95.8±5.6
	500	547.9±31.2		500	518.6±46.0
	5000	5354±318.1		5000	4937±177.5
	10 000	10 760±743.1		10 000	9856±295.0
2	*Quality control*		4	*Quality control*	
	5	4.7±0.3		5	5.8±0.8
	50	54.2±2.1		50	55.5±7.1
	500	526.4±32.9		500	521.2±20.2
	2500	2489±239.3		2500	2504±171.8
	5000	5136±153.9		5000	5473±150
	Dilution control			*Dilution control*	
	100	97.9±6.0		100	91.9±3.9
	500	520±38.6		500	546.7±51.9
	5000	5620±223.5		5000	5567±241.9
	10 000	10 220±355.0		10 000	11 630±264.2

the dilution control set had within-day RSD values less than 8.3% and between-day RSD values less than 7.4%. Zidovudine in the dilution control set had within-day RSD values less than 8.1% and between-day RSD values less than 7.2%.

The limits of quantitation determine the range of analyte concentrations that can be measured with acceptable accuracy and precision. The lower limit of quantitation for undiluted samples is 5 ng/ml for both lamivudine and zidovudine. The upper limit of

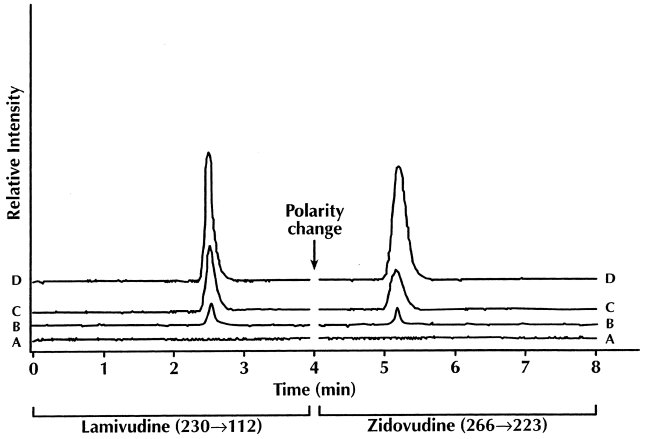

Fig. 2. Representative chromatograms of (A) a double blank human sample, (B) a 5 ng/ml quality control sample, (C) a 100 ng/ml dilution control, and (D) a diluted human clinical sample back-calculated to contain 1401.4 ng/ml lamivudine and 224.6 ng/ml zidovudine.

Table 4
Accuracy and precision of lamivudine analysis (RSD=relative standard deviation)

Concentration (ng/ml)	Accuracy (% Bias)	Between-assay precision (% RSD)	Within-assay precision (% RSD)
Quality control			
5	3.9	5.0	14.3
50	2.5	1.0	6.3
500	5.4	2.1	5.9
2500	1.5	4.3	6.1
5000	1.1	4.5	5.2
Dilution control			
100	3.1	5.8	7.0
500	4.7	1.0	8.2
5000	6.1	6.2	4.3
10 000	6.7	7.3	3.2

quantitation for undiluted samples is 5000 ng/ml for both drugs. Use of the dilution controls shows there is no deviation from linearity with a 2:1 dilution of sample containing between 100 ng/ml and 10 000 ng/ml of either analyte with ammonium acetate . Typical chromatograms at the lower limit of quantitation without dilution (5 ng/ml) and with ammonium acetate dilution (100 ng/ml) are shown in Fig. 2.

3.2. Stability testing

To be certain lamivudine and zidovudine were stable in human semen, a 9-month stability test was conducted. The spiked human sample from November 1998 was shown to be equivalent to the fresh spiked sample from August 1999. Following a two one-sided *t*-test, the 95% confidence interval constructed for lamivudine was from −2.4 to 12.4. The 95% confidence interval for zidovudine was −13.7 to 7.6. Both intervals were within ±15% of zero, ensuring the two samples to be equivalent in their lamivudine and zidovudine concentration and both compounds are stable in semen for at least 9 months.

3.3. Clinical data

Six HIV positive men, who were being administered lamivudine plus zidovudine combination antiviral therapy, volunteered to participate in a study to measure lamivudine and zidovudine in seminal

Table 5
Accuracy and precision of zidovudine analysis (RSD=relative standard deviation)

Concentration (ng/ml)	Accuracy (% Bias)	Between-assay precision (% RSD)	Within-assay precision (% RSD)
Quality control			
5	8.2	7.6	9.9
50	8.2	1.0	7.4
500	6.9	1.0	5.8
2500	3.1	2.3	7.2
5000	7.1	2.8	2.7
Dilution control			
100	4.9	3.2	5.5
500	6.7	1.0	8.0
5000	7.5	5.5	4.6
10 000	6.2	7.1	4.3

plasma. Seminal plasma from these men was collected and assayed using the above procedure. Typical chromatograms of clinical samples analyzed with ammonium acetate dilution are shown in Fig. 2. Samples were analyzed both with and without dilution with ammonium acetate.

Without dilution, the concentration of lamivudine ranged from 1431.6 ng/ml to above the limit of quantitation and the concentration of zidovudine ranged from 160 to 439 ng/ml (Table 6). Following dilution, the levels of lamivudine and zidovudine in semen ranged from 1401.6 to 5283.5 ng/ml and from 151.1 to 438.7 ng/ml, respectively. The RSDs between these six paired samples ranged from 0.8 to 7.0% for lamivudine and 0.7 to 6.7% for zidovudine.

The bioanalytical method is accurate and precise for the rapid quantitation of lamivudine and zidovudine in human seminal plasma. The method is sensitive and selective, with a dynamic range of 5 to 5000 ng/ml. More concentrated samples may be diluted without losing precision or accuracy. There is no interference from the seminal matrix when analyzing human samples, and samples can be safely stored for at least 9 months at −5°C.

Using a radioimmunoassay, Henry et al. first reported zidovudine levels in semen [20]. However, this HPLC–MS–MS method avoids the use of radioactive materials, uses a smaller sample size (100 μl versus 25 μl), requires less sample preparation, and is able to determine both lamivudine and zidovudine concentrations in a single analytical run. This method gives comparable clinical zidovudine semen concentrations to results published by Henry et al. [20]. The method described here is the first reporting seminal lamivudine concentrations. Research from a clinical study using concomitant semen and blood plasma samples from HIV-1 positive men prescribed lamivudine plus zidovudine combination therapy was recently published. The goal of the study was to determine the distribution of these drugs into the male reproductive tract. This method was used to quantitate the seminal concentrations of lamivudine and zidovudine in the patient samples [24]. Because lamivudine and zidovudine only act as antiviral agents when phosphorylated within cells, it is important to know what the phosphorylated drug levels are in the cell fraction of semen. Future work entails adapting this method to measure intra-cellular levels of phosphorylated lamivudine and zidovudine.

Table 6
Analysis of six paired clinical semen samples (N/A=not applicable, RSD=relative standard deviation)[a]

Patient		Undiluted	Diluted	Mean	% RSD
1	Lamivudine	3701.4	4087.8	3894.6	7.0
	Zidovudine	213.0	226.4	219.7	4.3
2	Lamivudine	2770.0	3003.6	2886.8	5.7
	Zidovudine	439.0	434.7	436.9	0.7
3	Lamivudine	1431.6	1401.4	1416.5	1.5
	Zidovudine	205.2	224.6	214.9	6.4
4	Lamivudine	3140.8	3177.0	3158.9	0.8
	Zidovudine	417.2	438.7	428.0	3.6
5	Lamivudine	2590.8	2452.1	2521.5	3.9
	Zidovudine	160.0	151.7	155.9	3.8
6	Lamivudine	>5000	5283.5	N/A	N/A
	Zidovudine	291.6	320.7	306.2	6.7

[a] The clinical samples were analyzed both with ammonium acetate dilution and without. Although only one sample here shows the need for dilution, approximately one-third of all seminal samples requires ammonium acetate dilution for exact quantitation.

4. Conclusions

In conclusion, a validated HPLC–MS–MS assay for simultaneous determination of lamivudine and zidovudine concentrations in human seminal plasma has been developed. This method offers several advantages over previous methodologies used to quantitate nucleoside analogs in biological matrices. The procedure requires small quantities of seminal plasma (as little as 25 μl), no radioactive material, minimal preparation, and short HPLC–MS–MS run times. The method offers excellent sensitivity and selectivity. It is likely that this assay could also be modified to quantitate other antiviral compounds in seminal plasma.

Acknowledgements

The authors thank Luigi Troiani from the University of North Carolina and Lisa St. John-Williams and Marty St. Claire from Glaxo Wellcome for their technical support. Funding was provided by NCDDG AI 33363-07, RO1 49381, UNC CFAR P30-HD37260, NIH Training Grant NIO 7001, and Glaxo Wellcome.

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