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Journal of Chromatography B, 766 (2002) 307–317

JOURNAL OF CHROMATOGRAPHY B

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Determination of amprenavir, a HIV-1 protease inhibitor, in human seminal plasma using high-performance liquid chromatography–tandem mass spectrometry

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Received 27 June 2001; accepted 16 October 2001

Abstract

A HPLC–MS–MS method to measure amprenavir in human seminal plasma has been developed and validated. The procedure uses stable, isotopically labeled $^{13}C_6$-amprenavir as an internal standard and 100 μl of sample. The method is accurate (bias less than or equal to 7.2%) and precise (within- and between-day RSDs less than or equal to 4.2%) over the dynamic range of 30–4000 ng/ml. Recently, this simple and sensitive method was used to determine amprenavir concentrations in seminal samples collected from HIV-1 positive subjects receiving amprenavir antiretroviral therapy as part of a multicenter clinical trial.

Keywords: Amprenavir

1. Introduction

Amprenavir (141W94, VX-478, Fig. 1) is the fifth HIV-1 protease inhibitor to become available for the treatment of HIV-1 (human immunodeficiency virus type-1) infected patients. At its recommended dosage, amprenavir reduces viral load in both seminal and blood plasma in most patients [1,2]. Because contact with semen is the major route for sexual transmission of HIV-1 [3], it is likely that drugs, which penetrate into male genital tract and reduce seminal shedding of HIV-1, will reduce the risk of sexual transmission.

Distribution of HIV-1 protease inhibitors into sanctuary sites has been reviewed [4,5]. Data suggest that ritonavir and saquinavir [6] do not distribute into the male genital tract, while indinavir [7] penetrates the genital barrier well. It is likely that the HIV-1 protease inhibitor amprenavir, which exhibits restricted penetration into the central nervous system, also has limited distribution into the male genital tract [8]. A better understanding of antiretroviral drug distribution into the male genital tract may lead

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1570-0232/02/$ – see front matter
PII: S0378-4347(01)00512-6

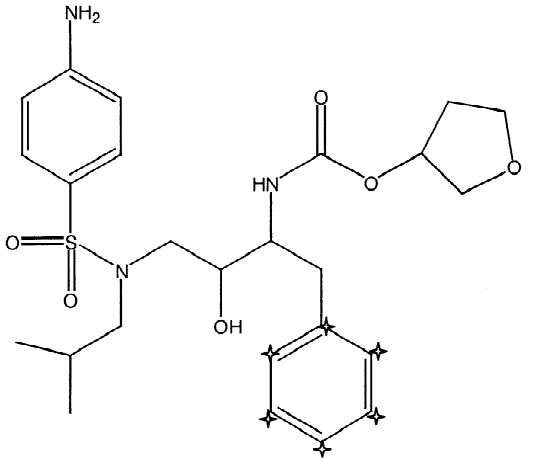

Fig. 1. Amprenavir and its $^{13}C_6$-internal standard (labeled carbons are highlighted). The internal standard is 6 u greater than the parent drug, amprenavir.

to effective therapies that reduce not only viral load in infected patients, but sexual transmission as well.

A recent MEDLINE search revealed at least 20 methods that have been published for the quantification of HIV-1 protease inhibitors in human blood plasma [9–29]. Five methods described quantification of HIV-1 protease inhibitors in other biological matrixes, including urine, cerebral spinal fluid, and semen [13,16,22,25,30]. The majority of the methods involve high-performance liquid chromatography (HPLC) with ultraviolet detection. Only three of these published methods employ mass spectrometry (MS), all of which involve quantitation of indinavir [11,28,30]. As antiretroviral regimens become more complicated, there is a growing need for multiple drug monitoring. In fact, at last count seven methods describe the determination of several HIV-1 protease inhibitors in a single analytical run [10,18, 19,21,24,26,27].

Amprenavir determination in blood plasma has been described in the literature by HPLC–fluorescence [25] and by HPLC–UV [19,24]. An abstract presented at a national meeting described the first validated HPLC–MS–MS method for measuring amprenavir in blood plasma [31]. Until now, only a partially validated method has been published for determining amprenavir concentrations in seminal plasma [25]. This earlier method lacked information concerning inter-assay precision and used blood plasma standards for calibration controls.

This article describes a full validation of a HPLC–MS–MS method for the quantifying amprenavir in human seminal plasma, using bull semen as calibration standard matrix and human semen as quality control sample matrix. This method, which uses $^{13}C_6$-labeled amprenavir as the internal standard, is the first validated assay to quantify amprenavir concentrations in seminal plasma. Recently, this method was used to support a multicenter clinical study examining the penetration of amprenavir into human seminal plasma.

2. Experimental

2.1. Materials

Amprenavir and its stable isotopically-labeled internal standard (Fig. 1), $^{13}C_6$-amprenavir, were prepared at GlaxoWellcome, now GlaxoSmithKline (Research Triangle Park, NC, USA and Stevenage, UK, respectively). Control human semen was collected, with informed consent, from HIV-1 negative, antiretroviral naive volunteers at UNC Hospitals (Chapel Hill, NC, USA). Bull semen, used for making calibration standards, was obtained free from antibiotics and other additives from Select Sires (Plain City, OH, USA). Clinical samples were obtained, with informed consent, from hospitals participating in a multicenter clinical study designed to assess amprenavir penetration into the male genital tract. HPLC-grade water, methanol, and acetonitrile and GR-grade formic acid were obtained from EM Science (Cincinnati, OH, USA). Snap-cap centrifuge vials were purchased from Fisher Scientific (Springfield, NJ, USA).

2.2. Chromatographic conditions

The primary mobile phase, which consisted of acetonitrile–water (55:45, v/v) with 0.1% formic acid, was used to elute the samples from an Aquasil (C_{18} 5 μm, 150×2.1 mm) analytical column

(Keystone Scientific, Bellefonte, PA, USA) at a flow-rate of 0.3 ml/min. The secondary mobile phase consisted of acetonitrile–water (55:45, v/v) with a flow-rate of 0.3 ml/min. A needle wash of acetonitrile–water (50:50, v/v) preceded each injection. The injection volume was 40 μl and sample elution was carried out at room temperature.

2.3. Instrumentation

The HPLC system (Fig. 2) consisted of a dual-pumping scheme controlled by a 10-port Valco switching valve (Valco Instruments, Houston, TX, USA). Mobile phase from the primary pump (Waters 616, Milford, MA, USA) was used to elute am-

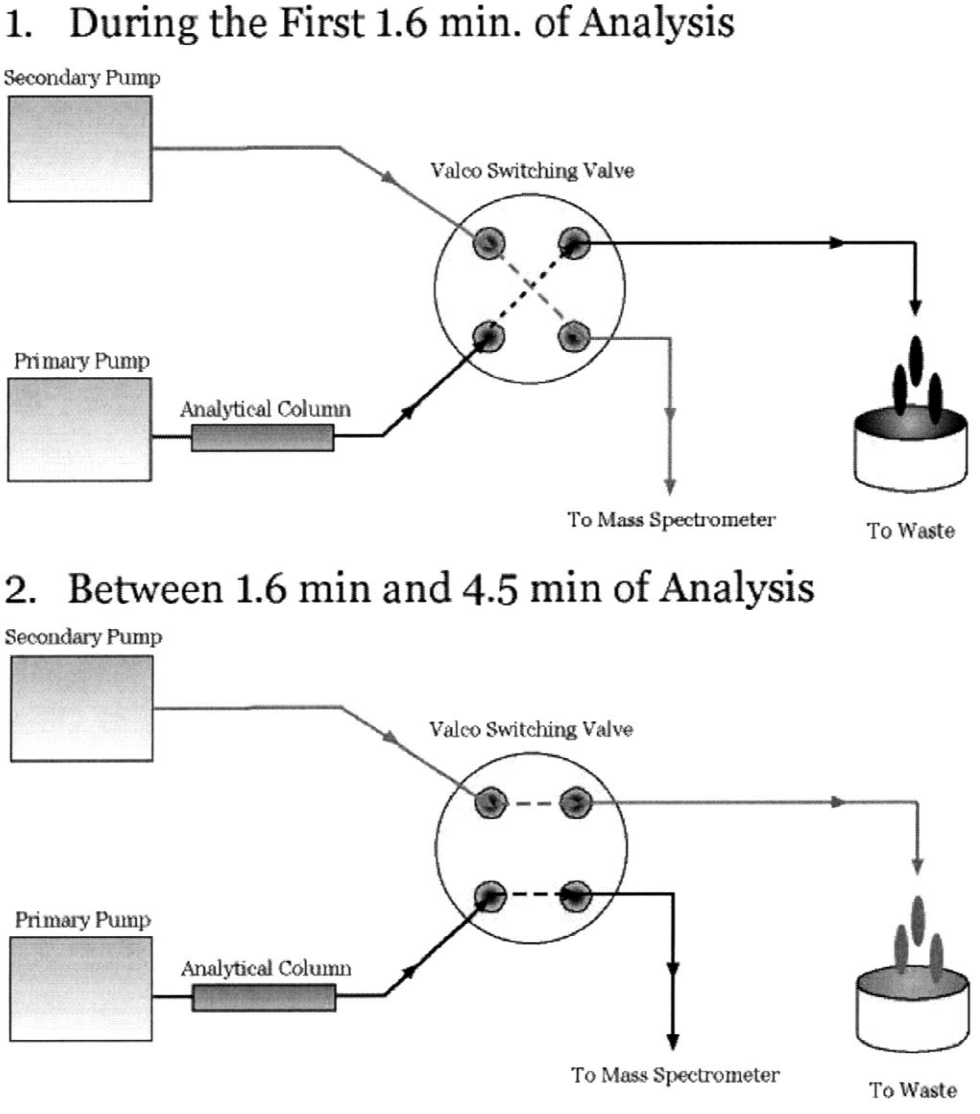

Fig. 2. During the first 1.6 min of analysis, mobile phase from the secondary pump flows directly to the mass spectrometer and mobile phase from the primary pump elutes the column to waste. Between 1.6 and 4.5 min of analysis, the valve reverses and the analytical column eluent flows to the mass spectrometer. After 4.5 min, the valve is reset for the next sample injection.

prenavir and $^{13}C_6$-amprenavir from the analytical column. Solvent from the secondary pump (Varian 9012, Palo Alto, CA, USA) bypassed the column and flowed to the mass spectrometer during the first 1.6 min of each analytical run. During this time, the switching valve diverted flow from the primary pump through the column to waste. After 1.6 min, the valve switched, and mobile phase from the primary pump eluted the column to the mass spectrometer while mobile phase from the secondary pump was diverted to waste. An API 300 triple quadrupole mass spectrometer (PE-Sciex, Norwalk, CT, USA) equipped with a TurboIonspray source was used to detect amprenavir and its stable isotopically-labeled internal standard.

2.4. Mass spectrometric conditions

The mass spectrometer was interfaced to the HPLC system using a TurboIonspray source (PE-Sciex) and analysis was conducted in the positive ion mode. Nitrogen was used as curtain, nebulizer and collision gases. User controlled voltages, gas pressures, and source temperature were optimized for the detection of the parent and product ions of amprenavir. The first quadrupole, Q1, was optimized for the maximum transmission of the protonated molecule of amprenavir (m/z 506). The third quadrupole, Q3, was optimized for the maximum transmission of the collision induced product ion of amprenavir (m/z 418). Both Q1 and Q3 were operated at unit mass resolution.

2.5. Data acquisition and analysis

Data acquisition was performed using multiple reaction monitoring (MRM) of amprenavir with its internal standard (Fig. 3). Transitions monitored were m/z 506→418 for amprenavir and m/z 512→424 for the $^{13}C_6$-labeled internal standard. The dwell time for each transition was 500 ms with a 5 ms pause between scans. Automated data acquisition and analysis were performed using the SampleControl application (PE-Sciex). Post-acquisition quantitative analyses were performed using MacQuan software (PE-Sciex). Unknown sample concentrations of amprenavir 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 amprenavir to its internal standard versus amprenavir concentration.

2.6. Semen pretreatment

Whole bull semen was pooled for use in preparing calibration standards and whole antiretroviral naive human semen was pooled for use in preparing controls. Clinical samples were kept in their separate aliquots. All semen was allowed to liquefy for 1 h at room temperature and then centrifuged for 30 min at 1000 *g*. The supernatant, or seminal plasma, was heated in a dry heat oven for 3 h at 58°C to inactivate HIV-1. Seminal plasma was kept frozen at −5°C until needed.

2.7. Preparation of stock and intermediate solutions

In order to prevent internally consistent, but inaccurate results, separate weighings were used to prepare independent calibration and quality control stock solutions, each containing 400 ng/μl of amprenavir in methanol. In order to reduce systematic errors associated with serial dilutions, aliquots of both stock solutions were diluted with water to form intermediate calibration and quality control stock solutions of 100, 50 and 10 ng/μl.

2.8. Preparation of calibration standards and control samples

Calibration standards, with amprenavir concentrations of 10, 30, 100, 300, 600, 1800, 4000 and 5000 ng/ml, were prepared in bull seminal plasma and were used to construct calibration curves. Quality control samples, containing 30, 800, 1800, and 4000 ng/ml amprenavir, were prepared in human seminal plasma and were used to measure the accuracy and precision of the assay throughout the entire procedure. Table 1 and Table 2 describe the preparation of calibration standards and control samples, respectively. Calibration standards, quality control samples, and clinical samples were stored at −5°C.

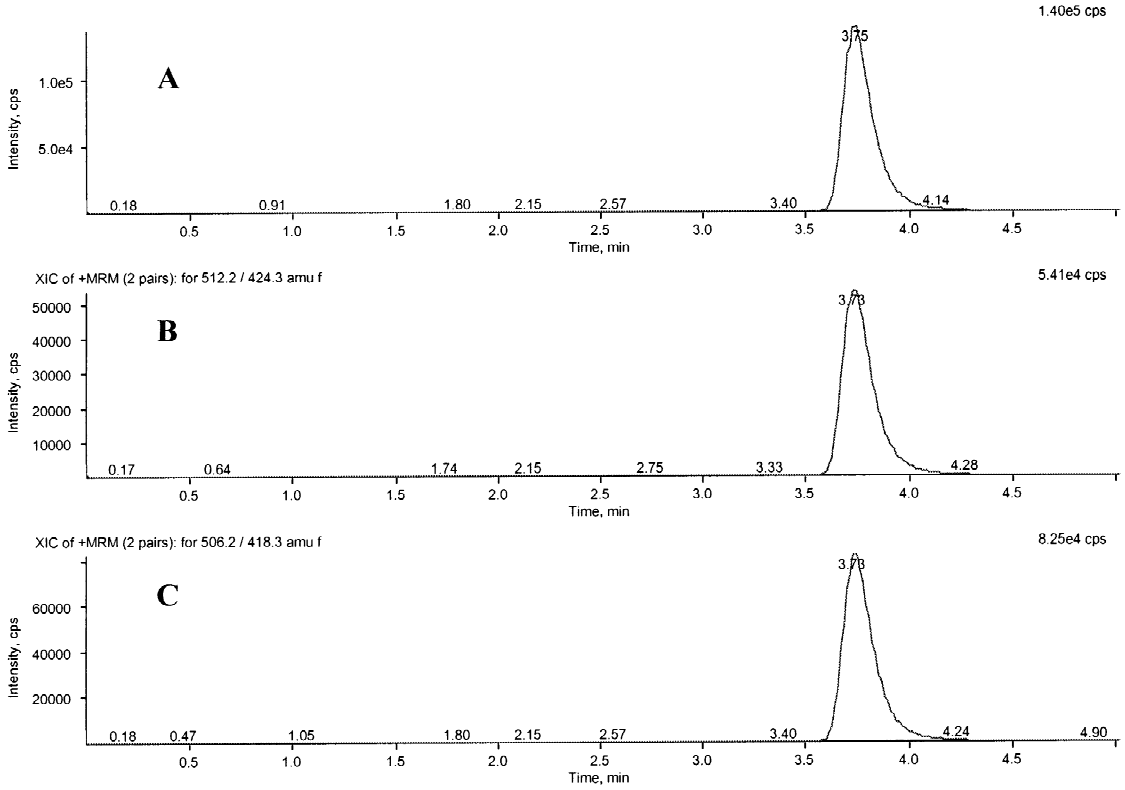

Fig. 3. Panel A describes the total ion current during amprenavir/amprenavir internal standard MRM. The peak is composed of amprenavir and its internal standard. Panels B and C reveal the amount $^{13}C_6$-internal standard (512→424) and amprenavir (506→418) in the total ion chromatogram, respectively.

2.9. Preparation of internal standard solution

A stock solution containing 20 ng/μl of $^{13}C_6$-amprenavir in methanol was prepared. This stock solution was stored refrigerated. The final, working solution of 500 ng/ml amprenavir internal standard was prepared fresh daily by diluting the stock solution with acetonitrile.

Table 1
Preparation of calibration standards

Nominal concentration (ng/ml)	Stock solution used (ng/μl)	Volume of stock solution taken (μl)	Total volume in bull semen (ml)
10	10	10	10
30	10	30	10
100	50	20	10
300	50	60	10
600	100	60	10
1800	100	180	10
2000	400	50	10
5000	400	125	10

Table 2
Preparation of quality control samples

Nominal concentration (ng/ml)	Stock solution used (ng/μl)	Volume of stock solution taken (μl)	Total volume in human semen (ml)
30	10	30	10
800	100	80	10
1800	400	45	10
4000	400	100	10

2.10. Sample preparation and analysis

First, 100-μl aliquots of each standard, control, and clinical sample were pipetted into separate snap-cap microcentrifuge vials containing 200 μl of working internal standard solution. Each tube was then vortex-mixed and refrigerated for 30 min. Next, the tubes were placed in a swinging-bucket centrifuge for 20 min at 5°C at 1000 *g*. Finally, a 100-μl aliquot of the supernatant from each tube was transferred to individual HPLC injection vials containing 100 μl 0.1% formic acid. The injection vials were vortex-mixed and placed in an automated sample injector. As described above, 40 μl of the supernatant–formic acid mixture was injected onto the analytical column.

2.11. Assay validation

Four separate analytical runs were used to determine the accuracy, precision, and specificity of the method. Each run contained calibration standards analyzed in duplicate, with one set of standards analyzed at the beginning and the other set at the end of the run. Each run also contained control samples analyzed in replicates of six to assess accuracy and precision, blank samples to assess specificity, and clinical samples to determine maximum batch size. Control, blank, and clinical samples were analyzed in random order.

2.11.1. Specificity

Specificity of the method for amprenavir and its $^{13}C_6$-internal standard was tested to ensure that no endogenous co-eluting peaks would interfere with the quantitation of amprenavir. Protein precipitation of blank bull and human seminal samples was carried out with internal standard free acetonitrile and chromatographed as described above.

2.11.2. Calibration model

In order to prevent under or over estimation of the true value of unknown samples, it is important to select the proper mathematical relationship (calibration model) 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 before validation. The appropriate weighting scheme (unweighted, $1/x$, $1/x^2$) was determined by constructing calibration lines using results from the four analytical runs under the influence of the three different weighting schemes. The percentage error (%bias) between nominal and back calculated concentration of each calibration standard was then determined under each scheme. By adding the absolute values of the %bias under the three different weighting schemes, three different total errors were calculated for each analytical run. Grand total errors were calculated for each of the three schemes by adding the total error for all four analytical runs. The weighting factor that gave the lowest grand total error over the four runs was chosen and used during this validation procedure and subsequent analytical runs.

2.11.3. Accuracy

Accuracy describes the agreement between measured and nominal concentrations. Control samples analyzed during the four consecutive analyses were used to calculate the accuracy at each control concentration. In all, 24 replicates of each control concentration were analyzed (six replicates per day for 4 days). The mean concentration of all 24 control samples was determined and used to calculate the %bias for each control concentration. For method

validation acceptance, the bias must be less than or equal to ±15% for each control concentration.

2.11.4. Precision

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

2.11.5. Limits of quantitation

The limits of quantitation designate the range of concentrations for which acceptable accuracy and precision have been demonstrated. Human seminal controls as low as 30 ng/ml and as high as 4000 ng/ml were tested for accuracy and precision, as described above. Bull seminal standards, which were only analyzed in duplicate, and were tested for accuracy from 10 to 5000 ng/ml

2.11.6. Stability testing

One analytical run was conducted to ensure analyte stability in matrix. Six aliquots of an 1800 ng/ml amprenavir spiked human seminal sample were prepared and stored, as described above. These stored aliquots were allowed to undergo multiple freeze–thaw cycles over 11 months before analysis in tandem with six aliquots of a freshly prepared 1800 ng/ml amprenavir spiked human seminal sample. In order to demonstrate long-term stability, a two-sample one-sided *t*-test (TOST) was performed at a 95% degree of certainty to determine whether amprenavir concentrations in the fresh and stored samples differed by more than 10%. The TOST was first described by Timm et al. in 1985 [32]. Briefly, the procedure finds a 90% confidence interval (CI) for the true mean difference between the fresh and stored samples. The fresh and stored samples are equivalent, and thus the amprenavir can be considered stable at −5°C for 11 months, if the entire CI falls within 10% of the mean reference (freshly prepared sample) concentration.

3. Results

3.1. HPLC–MS–MS assay validation

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

3.1.1. Specificity

Specificity of this method was examined using drug-free human and bull seminal plasma without the addition of any stock or internal standard solutions. No interfering endogenous materials were found in either the bull or human blank samples. A typical blank human chromatogram is shown in Fig. 4A.

3.1.2. Calibration model

To determine the appropriate calibration model to describe the relationship between the peak area ratio of analyte:internal standard and the analyte concentration, calibration curves were created and evaluated using three different weighting factors. The smallest grand total absolute error, tabulated over all four analytical runs, was obtained using a $1/x^2$ weighting factor. Typical r^2 values, when determined using the $1/x^2$ weighting factor, were greater than 0.995. The weighted calibration curve created for amprenavir was consistently linear. Typically, the calibration curve was defined by a slope of 0.001 and a *y*-intercept of −0.001. A $1/x^2$ weighting model was used throughout assay validation.

Table 3
Daily mean concentrations of amprenavir controls

Day	Concentration (ng/ml)	Mean±SD	Day	Concentration (ng/ml)	Mean±SD
1	30	33.3±5.7	3	30	31.6±4.3
	800	789±23.3		800	809.8±21.8
	1800	1846.1±46		1800	1886.3±35
	4000	4107.2±66.8		4000	4044.2±105.0
2	30	32.3±3.3	4	30	31.4±4.8
	800	812.2±32.1		800	796.5±27.1
	1800	1831±32		1800	1821.9±20
	4000	4116.5±79.3		4000	4052.8±71.8

3.1.3. Accuracy

The overall accuracy, using all 24 replicates, was determined for each control sample (Table 4). The accuracy of all control concentrations fell within the acceptable accuracy range (bias≤±15%). The greatest bias was 7.2% at 30 ng/ml.

3.1.4. Precision

All 24 replicates of each control concentration were used to determine within- and between-day precision (Table 4). The within- and between-day precision of all control concentrations fell within the acceptable precision range (RSD≤15%). The largest variation occurred at 30 ng/ml with a within-day RSD of 4.2% and a between-day RSD of 3.7%. A typical chromatogram of a 30 ng/ml control sample is shown in Fig. 4B.

3.1.5. Limits of quantitation

The limits of quantitation determine the range of analyte concentrations that can be measured with acceptable accuracy (bias was less than ±15%) and precision (RSD was less than 15%). Amprenavir concentrations as low as 30 ng/ml and as high as 4000 ng/ml in human controls fell within the 15% limits for bias and intra- and inter-day RSDs.

3.1.6. Stability testing

To be certain amprenavir is stable in human semen, an 11-month stability test was conducted. A two sample one-sided t-test (TOST) demonstrated that a spiked human seminal sample containing 1800 ng/ml amprenavir prepared in August 1999 and allowed to undergo multiple freeze–thaw cycles was equivalent to a freshly spiked human seminal sample containing 1800 ng/ml amprenavir. The 90% CI (constructed at a 95% degree of certainty) describing the true mean difference between the stored and fresh samples was contained within 10% of the freshly prepared mean. Thus, the stored and freshly prepared samples were equivalent in amprenavir concentration and amprenavir was stable in semen for at least 11 months despite multiple freeze–thaw episodes.

4. Discussion

The method described here was based upon previous work performed by Kenney and Dunn [31], which described a method to quantify amprenavir in human blood plasma using tandem mass spectrometry. Because large volumes of antiretroviral naïve human seminal plasma were difficult to obtain, bull seminal plasma was used as a surrogate matrix for calibration standards. Because it is likely that the matrix effects on ionization described by Fu et al. are not limited to urine [30], substitution with blood plasma or other matrix may not be adequate. There is precedent for substituting bull semen for human semen [33], and human amprenavir controls analyzed using bull amprenavir calibration standards demonstrated acceptable accuracy and precision.

This method demonstrated acceptable precision and accuracy using human controls from 30 to 4000 ng/ml. Because accuracy of bull standards from 10 to 5000 ng/ml was greater than 85%, it is likely that this method is capable of analyzing human samples

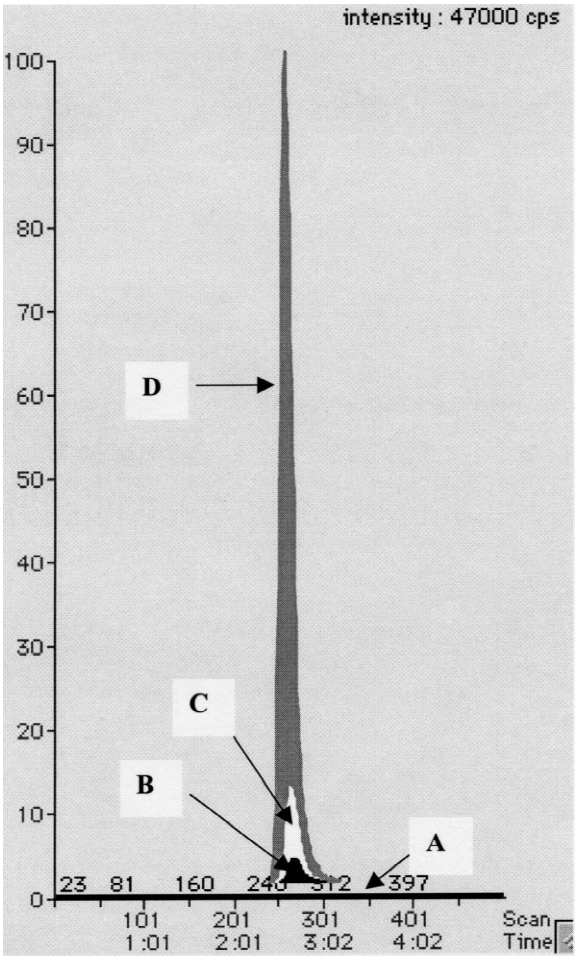

Fig. 4. All chromatograms are shown in the same relative scale. A (black line), B (black peak), C (white peak), and D (gray peak) are selected chromatograms from a typical analytical run of amprenavir. B, C, and D have been artificially shifted up from the *x*-axis so that A is visible. These chromatograms represent human blank, 30 ng/ml control, 100 ng/ml calibration standard, and 800 ng/ml control samples (A, B, C, D, respectively). Lack of peaks in A demonstrates method specificity. Median amprenavir concentration in the seminal plasma of men receiving 1200 ng amprenavir twice daily is 319 ng/ml.

from 10 to 5000 ng/ml, as well. It may be possible to measure amprenavir concentrations less than 10 ng/ml if sample supernatant were dried following protein precipitation and re-suspended in less than 300 μl acetonitrile–formic acid (1:1, v/v) or if a larger sample size was available for analysis.

A recent study analyzed amprenavir concentrations in 43 seminal samples obtained from 31 subjects receiving 1200 ng amprenavir twice daily [34]. The median seminal amprenavir concentration in these samples was 319 ng/ml. Subjects donated samples randomly throughout the dosing interval; thus, peak and trough concentrations were represented. All samples fell between 10 and 5000 ng/ml, two samples were less than 30 ng/ml and one was greater than 4000 ng/ml. Therefore, the dynamic range of 30–4000 ng/ml amprenavir was useful in most instances.

The HPLC–MS–MS method described here offers several advantages over previously published methodologies used to quantify HIV-1 protease inhibitors in biological matrices including: smaller sample size, no extensive sample preparation, use of an internal standard, and short HPLC–MS–MS run times. For example, four published methods for quantifying HIV-1 protease inhibitors did not use internal standards [13,16,19,27]. Analysts employing these methods risk encountering increased and uncompensated for user error.

While this method required only 0.1 ml sample, several methods require as much as 1 ml sample for analyzing a single HIV-1 protease inhibitor [9–11,22,29]. Because the average ejaculate contains approximately 2.5 ml of seminal plasma, additional routine analyses on a single seminal sample would be limited if 1 ml were reserved for monitoring a single drug. For example, a minimum 0.2 ml is required for measuring HIV-1. However, 1–2 ml is required for more sensitive HIV-1 assays (less than 50 copies/ml). Furthermore, additional seminal plasma is required if seminal virus is to be sequenced or if other antiretroviral drugs are to be analyzed.

This method, which required 4.5 min/sample for analysis, had the shortest runtime of all published methods for measuring HIV-1 protease inhibitors, including the method published by Sparidans et al. for the analysis of amprenavir in human plasma [25]. Only four others demonstrated run times less than 15 min [11,12,14,18] and four required over 30 min/sample [13,24,26,27].

The simple protein precipitation described in this method required less than 2 h, allowing more samples to be processed in a shorter period. Only two other methods for HIV-1 protease inhibitor quantification use this separation technique [14,16].

Table 4
Accuracy and precision of amprenavir analysis

Control concentration (ng/ml)	Accuracy (bias, %)	Between-assay precision (RSD, %)	Within-assay precision (RSD, %)
30	7.2	3.7	4.2
800	0.2	0.4	1.8
1800	2.6	1.3	2.1
4000	2.0	0.5	1.9

Most rely on more tedious solid-phase [9,10,13,18–20,22,24,27,28] and liquid–liquid [11,12,15,17, 21,23,26,29] extraction techniques. A long sample preparation time can cancel the benefits of a short runtime. For example, the method published by Ha et al. has a 10 min runtime, but relies on liquid–liquid extraction. Consequently, only 50 samples/day could be processed and analyzed [12]. The method described here was validated by processing and analyzing 96 samples/day. As demonstrated by HPLC–MS–MS methods described for indinavir [28] and amprenavir [31], with automation this method may be capable of analyzing hundreds of samples/day without loss of accuracy or precision.

It is likely that this HPLC–MS–MS method may be modified to determine concentrations of multiple antiretroviral drugs. In fact, the same instrumentation as described here has been used to measure indinavir concentrations in blood plasma and urine [11,30] and the nucleoside reverse transcriptase inhibitors zidovudine and lamivudine in blood [35] and seminal plasma [33].

5. Conclusion

In conclusion, a HPLC–MS–MS method, which requires only 100 μl of sample for the quantitation of amprenavir in human seminal plasma, has been developed. Through standard validation testing, this method was shown to have acceptable specificity, precision, and accuracy. The method is sensitive and selective, with a dynamic range of 30–4000 ng/ml. Based on stability studies, samples can be safely stored in matrix for at least 11 months at −5°C. The median seminal amprenavir concentration of 31 men enrolled in a multicenter clinical trial receiving 1200 mg amprenavir bid, was 319 ng/ml, well within the upper and lower limits of quantitation [34].

Acknowledgements

This publication resulted in part from research supported by GlaxoSmithKline Inc., the University of North Carolina at Chapel Hill Center for AIDS Research (CFAR, an NIH funded program 9P30 AI50410), and an NIH Training Grant (NIO 7001) to A.S.P.

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