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# Simultaneous determination of lamivudine, stavudine and nevirapine in human plasma by LC–MS/MS and its application to pharmacokinetic study in clinic

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**ABSTRACT: A new high-throughput LC–MS/MS method for the simultaneous determination of lamivudine (3TC), stavudine (d4T) and nevirapine (NVP) in human plasma is presented, with zidovudine as an internal standard. The analytes were extracted from plasma by protein precipitation and only 150 μL plasma was needed. Chromatographic separation was achieved on a Shiseido $C_8$ column (150 × 2.0 mm, 5 μm) with a total run time of 6 min. A tandem mass spectrometric detection was conducted using multiple reaction monitoring under positive ionization mode with an electrospray ionization interface. The method was developed and validated over the concentration range of 25–5000 ng/mL for 3TC and NVP and 20–4000 ng/mL for d4T. The method was validated in terms of intra- and inter-day precision (≤8.6%), accuracy (within ± 8.4%), linearity and specificity. The method has been successfully applied to the pharmacokinetic study of a combination treatment of 300 mg lamivudine, 30 mg stavudine and 200 mg nevirapine in 22 healthy male volunteers under fasting conditions. Copyright © 2010 John Wiley & Sons, Ltd.**

**Keywords:** lamivudine; stavudine; nevirapine; LC–MS/MS; pharmacokinetic study

## Introduction

Acquired immune deficiency syndrome (AIDS) is a very serious disease and millions of people infected with human immunodeficiency virus (HIV) die every year. Recently, many new drugs against HIV have gone on sale in the market. The common therapeutic strategy for treating HIV infection is administering three or four different antiretroviral drugs in combination (Gehrig *et al.*, 2007).

Highly active antiretroviral therapy (HAART) is one kind of effective combination generally for treatment of AIDS. It contains two nucleoside reverse transcriptase inhibitors (NRTI) and a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a single protease inhibitor (PI) (Gallant, 2002).

Lamivudine (3TC) and stavudine (d4T) are both NRTIs, and can inhibit the replication of HIV-1 and HIV-2 in different kinds of cells. Nevirapine (NVP), a kind of NNRTI used frequently, can idio-inhibit reverse transcriptase of HIV-1 (HIV-1 RT) (Jiang *et al.*, 2000).

Several methods have been described in the literature for simultaneous determination of 3TC, d4T and NVP in biological matrices. Aymard *et al.* (2000) and Notari *et al.* (2006) have reported simultaneous determination of these three drugs with other NRTIs, NNRTIs and PIs in human plasma by HPLC-UV with a run time of 40 or 35 min and volume of plasma of 1 or 0.6 mL. LC-MS/MS methods for simultaneous quantification of 3TC, d4T and NVP in human plasma were described by Narang *et al.* (2005), and Mistri *et al.* (2007) used a $C_{18}$ reversed-phase column and acid mobile phase with solid-phase extraction (SPE). The lower limit of quantitation (LLOQ) was from 51 to 20 ng/mL.

In this article, we describe a new method for the simultaneous determination of 3TC, d4T and NVP in human plasma and its application to pharmacokinetic study. An electrospray ionization (ESI) interface operated in positive ionization mode was used for multiple reaction monitoring (MRM) LC–MS/MS analyses. $C_8$ reversed-phase chromatography column was used to obtain better separation for all the analytes. We chose neutral acetate mobile phase and infused acetic acid aqueous solution after the chromatographic column. Protein precipitation was used for sample preparation with 150 μL human plasma. The LLOQ for 3TC, d4T and NVP was 25, 20 and 25 ng/mL, respectively.

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**Abbreviations used:** 3TC, lamivudine; AIDS, acquired immune deficiency syndrome; AZT, zidovudine; d4T, stavudine; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; HIV-1 RT, reverse transcriptase of HIV-1; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NVP, nevirapine; PI, protease inhibitor; PPT, protein precipitation.

## Experimental

### Chemicals and Reagents

Lamivudine (3TC, purity: 99.07%, batch no. 010RWSA0801), stavudine (d4T, purity: 99.92%, batch no. 021RWSA0801) and nevirapine (NVP, purity: 99.67%, batch no. 027RWSA0701) were all obtained from Desano Co. (Shanghai, China). Zidovudine (AZT, used as internal standard, IS, purity: 99.5%) was obtained from Shanghai Modern Pudong Pharmaceutical Factory Co. (Shanghai, China). HPLC-grade methanol was product of Sigma–Aldrich (Steinheim, Germany). Analytical grade acetic acid was obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Analytical-grade ammonium acetate was obtained from Shanghai No. 4 Chemical Reagent Factory (Shanghai, China). Purified water used throughout the study was commercially available (Wahaha®, Hangzhou Wahaha Co. Ltd, China).

### Chromatographic Conditions

An HPLC system (Shimadzu Co., Kyoto, Japan), consisting of two LC-10ADvp pumps, a SIL-HTc auto sampler, a CTO-10Avp column oven and a Shiseido $C_8$ column (150 × 2.0 mm, 5 μm, Shiseido Co. Ltd, Chuo-ku, Tokyo, Japan) equipped with a guard column ($C_{18}$, 4 × 3.0 mm, Phenomenex Co. Ltd, Torrance, CA, USA) was used for the chromatographic separation.

Mobile phases 'A' and 'B' consisted of methanol–water (80:20, v/v) and water, respectively, both containing 10 mM ammonium acetate. An elution profile comprising an isocratic step of 80% 'A' for 3 min and then 30% 'A' for next 3 min was used. An on-line motorized six-port divert valve was used to introduce the LC eluent flow into the mass spectrometer over the period of 2.5–5.8 min, while the other eluent flows were diverted to waste. The flow rate of mobile phase was maintained at 0.2 mL/min throughout the run. A Harvard '11' plus syringe pump (Harvard Apparatus Inc., MA, USA) was used to deliver a constant flow (2 μL/min) of 40% acetic acid aqueous solution into the mass spectrometer ionization source after the chromatographic column.

The temperatures of the analytical column and auto sampler were maintained at 30 and 5°C, respectively. Under these conditions, the retention times for 3TC, d4T, NVP and the internal standard were 3.1, 3.5, 5.2 and 4.3 min, respectively. The total run time for a HPLC–MS/MS analysis was 6 min.

### Mass Spectrometric Conditions

An API 3000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Ontario, Canada) with an electrospray ionization (ESI) interface operated in positive ionization mode was used for the multiple reaction monitoring (MRM) LC–MS/MS analyses. The operation conditions were optimized by infusing diluted stock solutions of each analyte as follows: nebulizing gas flow was 14 L/min, curtain gas flow was 12 L/min, collision gas flow was 6 L/min, source temperature was set at 300°C. The specific parameters for each analyte are shown in Table 1.

### Preparations of Standards and Quality Control Samples, and Internal Standard

Three separate primary stock solutions for 3TC, d4T and NVP were prepared in methanol at 1 mg/mL and stored at –20°C before use. The stock solutions were mixed together and serially diluted with methanol–water (20:80, v/v) to produce a series of standard or quality control (QC) working solutions at the desired concentrations.

The calibration standards were freshly prepared by spiking blank plasma with working solutions to provide the final concentrations of 25, 50, 200, 400, 1000, 2500 and 5000 ng/mL for 3TC and NVP, respectively and 20, 40, 160, 320, 800, 2000 and 4000 ng/mL for d4T.

Low-, medium- and high-level QC samples were prepared at 50, 400 and 4000 ng/mL for 3TC and NVP, respectively and 40, 320 and 3200 ng/mL for d4T.

Zidovudine (IS) was prepared as a stock solution of 100 μg/mL in methanol and diluted with methanol to yield a working standard of 500 ng/mL.

All solutions except the stock solutions described above were stored at 4°C.

### Sample Preparation

A volume of 150 μL thawed human plasma was transferred to a 1.5 mL Eppendorf tube and 60 μL of IS working solution was spiked and briefly vortex-mixed. To each sample, 400 μL of methanol was added for protein precipitation, and the tubes were capped and vortexed for 3 min, then centrifuged at 10,000***g*** for 5 min. The upper supernatant was carefully transferred to a glass tube, and evaporated to dryness under a gentle stream of nitrogen in water bath at 50°C. The residue was reconstituted with 300 μL of methanol–water (20:80, v/v), and an aliquot of 10 μL was injected into the LC–MS/MS system for analysis.

### Method Validation

The current LC–MS/MS assay was validated for specificity, linearity, intra-day and inter-day precision, accuracy and stability. Selectivity of the method was confirmed by analyzing different lots of blank plasma without the presence of internal standards.

Three validation batches, each containing two set of calibration standards and five replicates of QC samples at low, medium and high concentration levels, were assayed to assess the linearity, intra- and inter-day precision and accuracy of the method.

The linearity of each curve was confirmed by plotting the peak area ratio ($y$) of the analyte to IS vs analyte concentration ($x$). The accuracy of the assay was expressed by [(mean observed concentration)/(spiked concentration)] × 100% and precision was evaluated by relative standard deviation (RSD).

The stability of 3TC, d4T and NVP in spiked samples was investigated. The stability experiments aimed at testing the effects of possible conditions that the analytes might experience during collection, storage and analysis, including three cycles of freeze and thaw, stored frozen at –20°C for 80 days, stored at room temperature for 4 h, and storage of extracted samples in an autosampler (5°C) for 24 h.

**Table 1.** Optimized mass parameters for lamivudine, stavudine, nevirapine and the internal standard

| Analyte | MRM ($m/z$) | Dwell time (ms) | DP(V) | CE(V) | FP(V) | EP(V) | CXP(V) |
|---|---|---|---|---|---|---|---|
| Lamivudine | 230.0 → 112.1 | 100 | 45 | 18 | 300 | 10 | 10 |
| Stavudine | 225.0 → 127.0 | 100 | 38 | 13 | 300 | 5 | 10 |
| Nevirapine | 266.9 → 226.3 | 100 | 48 | 34 | 300 | 10 | 10 |
| Zidovudine(IS) | 267.9 → 127.0 | 100 | 30 | 18 | 300 | 10 | 10 |

DP, declustering potential; CE, collision energy; FP, focalization potential; EP, entrance potential; CXP, collision cell exit potential.

### Matrix Effects and Recovery

To study the matrix effects, six different lots of blank plasma were extracted and then spiked with each analyte and IS on the QC's concentration in parallel. Absolute matrix effects were estimated by the post-extraction addition method. Matrix effects (ME%) was calculated by comparing the peak areas of analytes added post-extraction in six different lots of plasma (B) with mean peak areas of standard at the same concentrations (A), and expressed as (B/A × 100%). Tests were conducted in five replicates on a single lot of plasma. ME% of 100% indicates that there is no absolute matrix effect, ME% > 100% a signal enhancement and, correspondingly, ME% < 100% a signal suppression.

Relative matrix effects was used to evaluate the variations of different lots of plasma suffered from the matrix effects, and was calculated by the coefficients of variation (CV%) of peak areas of analyte added post-extraction from five different lots of blank plasma.

Recovery presents the extraction efficiency of a method. It was performed at low, medium and high QC levels and evaluated by comparing peak areas of QC samples (C) with B, expressed as (C/B × 100%).

## Results

### Specificity and Selectivity

Abundant protonated molecules of 3TC, d4T and NVP that formed the base peak of each mass spectrum were observed from Q1 scans during the infusion of the neat solution in positive mode.

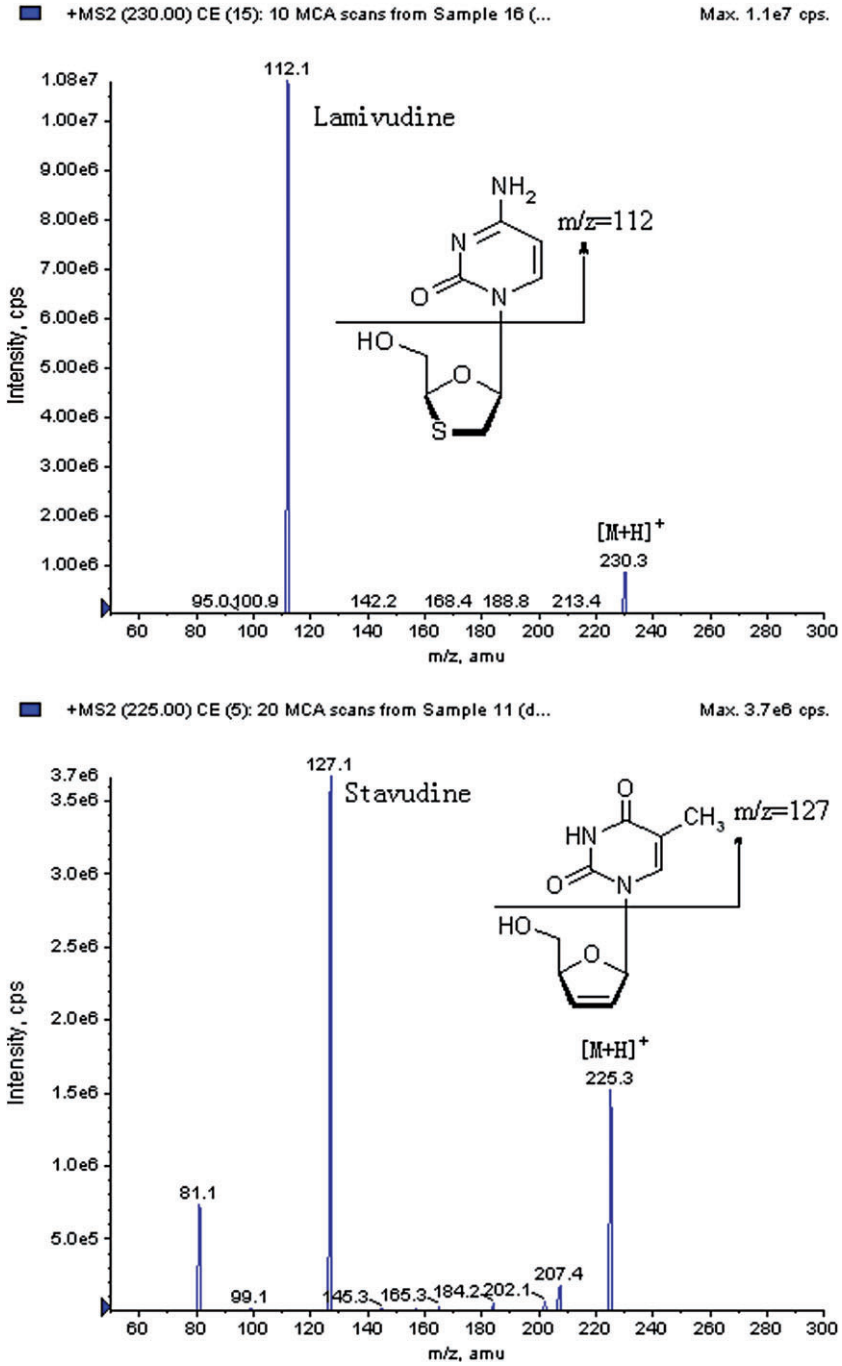


**Figure 1.** MS/MS product ion mass spectra of the protonated molecules $[M + H]^+$ of lamivudine, stavudine, nevirapine and zidovudine (IS).

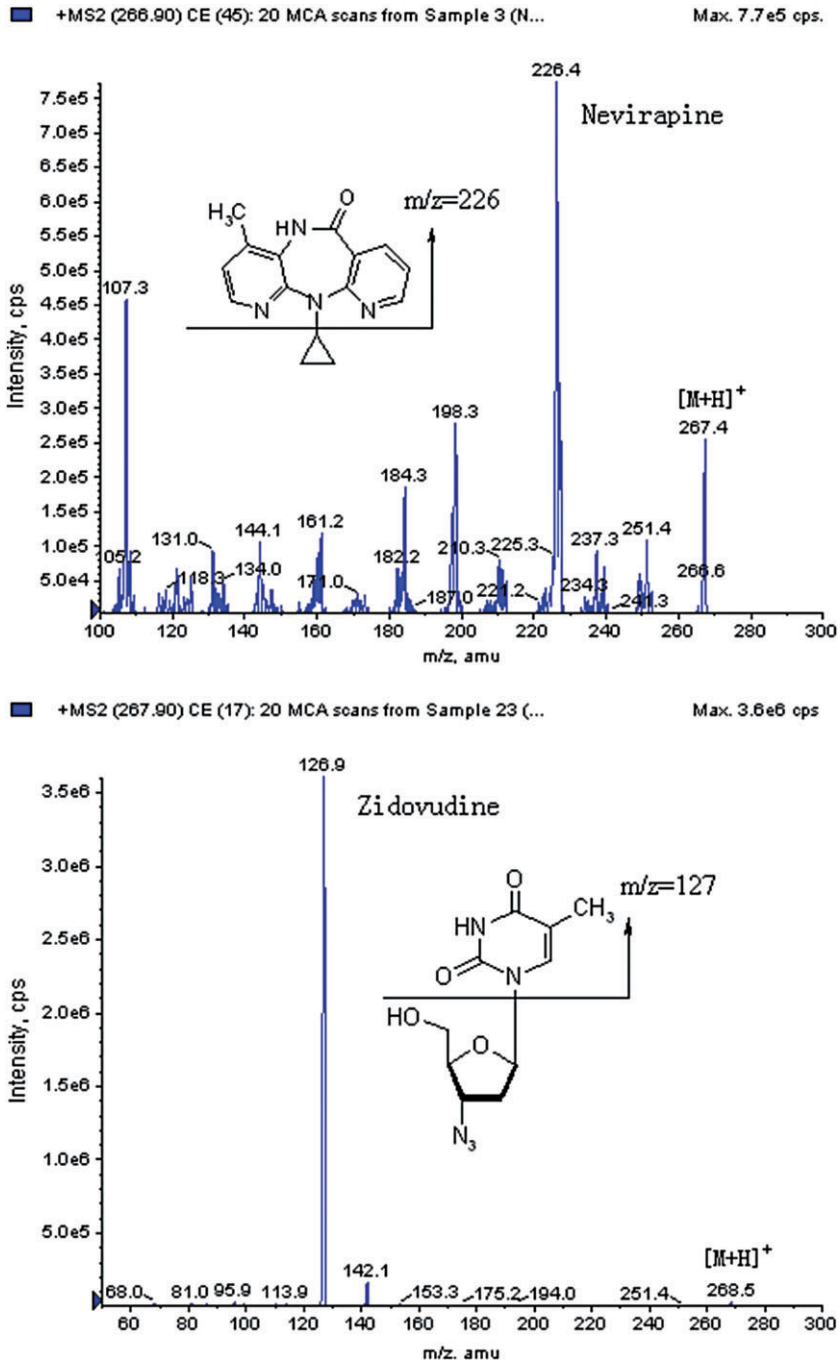


**Figure 1.** *Continued.*

Three $[M + H]^+$ precursor ions, *m/z* 230.0 for lamivudine, *m/z* 225.0 for stavudine and *m/z* 266.9 for nevirapine, were subjected to collision-induced dissociation (CID). The product ion tandem mass spectra of the protonated molecules of 3TC, d4T and NVP are shown in Fig. 1. Mass transition patterns, *m/z* 230.0 → 112.1, *m/z* 225.0 → 127.0 and *m/z* 266.9 → 226.3 were selected to monitor 3TC, d4T and NVP, respectively. A MS/MS channel of *m/z* 267.9 → 127.0 was chosen to monitor the internal standard, zidovudine.

Under the current LC–MS/MS conditions, the three analytes were well separated from interferences in the matrix. Chromatograms of different lots of blank plasma were found to contain no endogenous peak co-eluted with any of the analytes or the internal standard. Representative chromatograms of blank samples with or without the presence of analytes and internal standard are shown in Fig. 2. In addition, the 'cross-talk' between channels used for monitoring the analytes and IS was evaluated by analysis of their individual solution at high concentration. The responses in the MRM mass transition channels used for quantification were monitored. No 'cross-talk' or interference between the analytes and IS was observed.

### Matrix Effects and Recovery

The results of the matrix effects investigations are shown in Table 2. Absolute matrix effects demonstrated that there was no evident matrix effect on NVP and there was MS signal

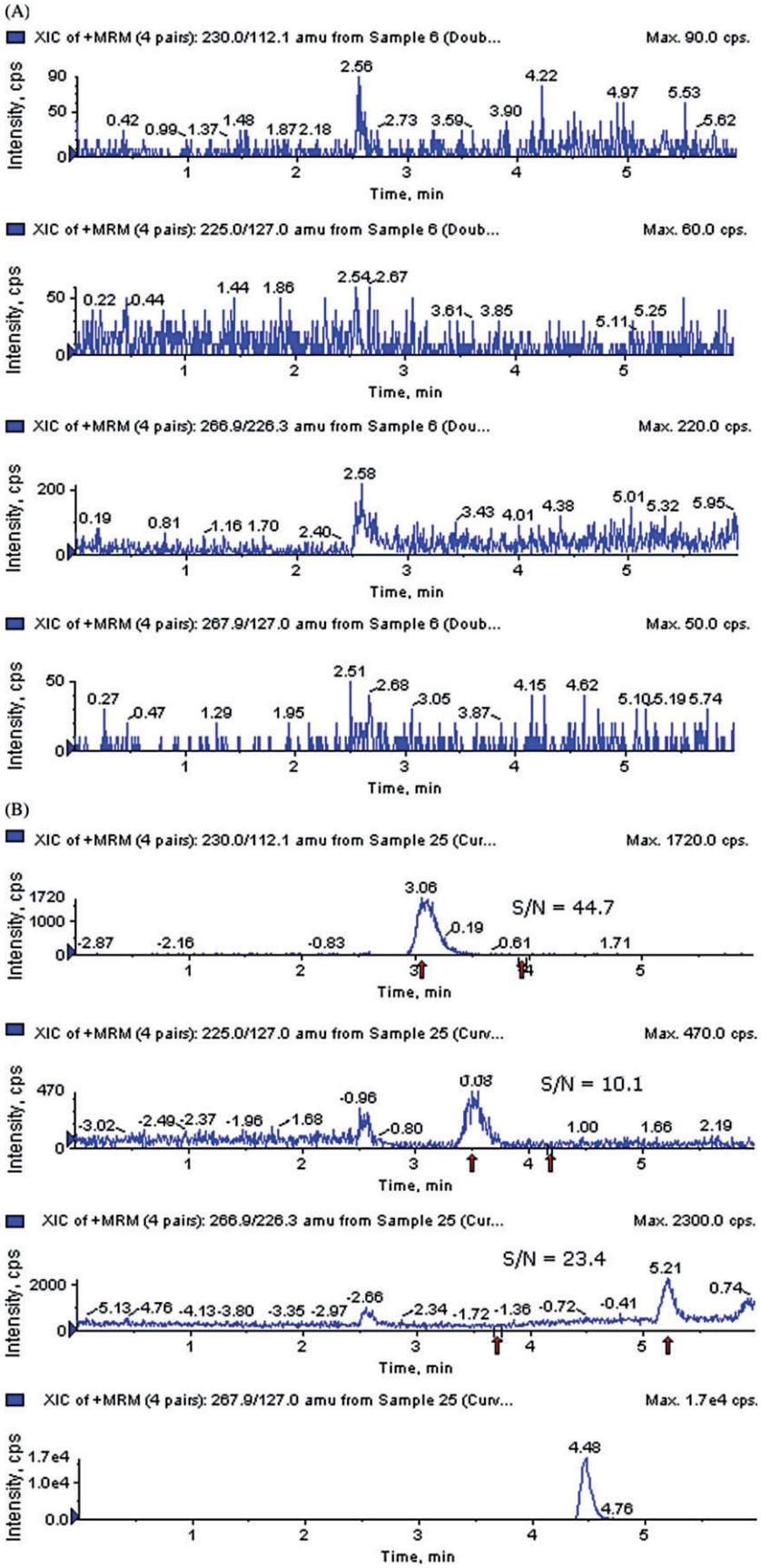


**Figure 2.** Representative MRM chromatograms for (A) blank plasma, (B) LLOQ, 25 ng/mL for 3TC, NVP, 20 ng/mL for d4T and 100 ng/mL AZT in plasma (C) plasma sample 2 h after administration of 3TC (300 mg), d4T (30 mg) and NVP (200 mg).

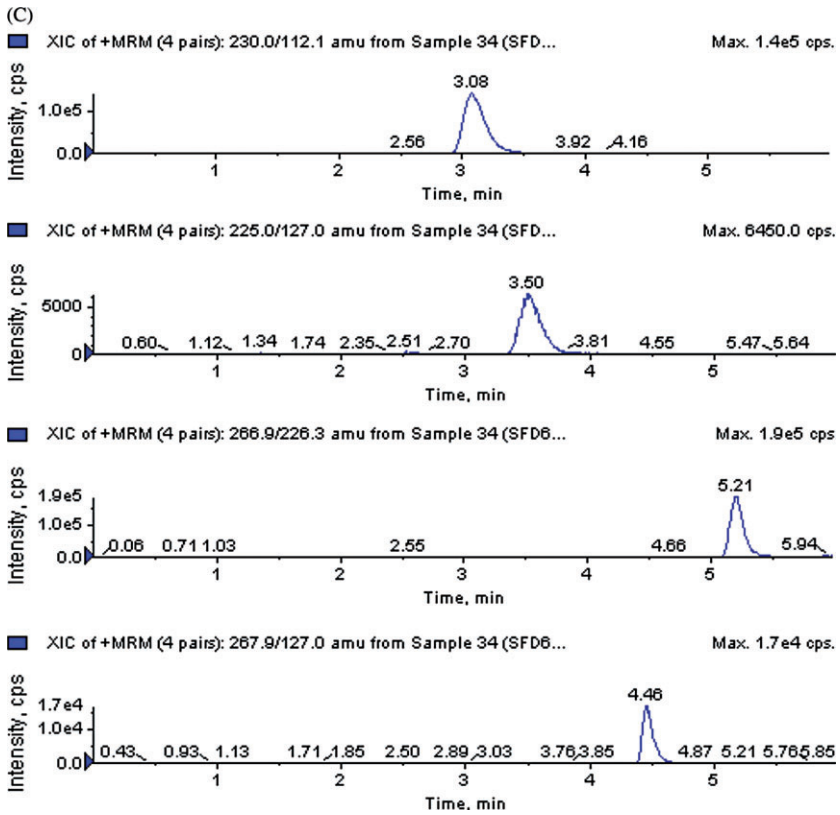


**Figure 2.** *Continued.*

**Table 2.** Matrix effects for 3TC, d4T, NVP and internal standard in six different lots of human plasma ($n = 6$)

| Analyte | Analyte concentration (ng/mL) | Matrix effects (%) | CV (%) |
|---|---|---|---|
| 3TC | 50 | 74.6 ± 1.7 | 2.3 |
| | 400 | 78.7 ± 2.2 | 2.8 |
| | 4000 | 83.5 ± 0.8 | 1.0 |
| d4T | 40.8 | 80.0 ± 3.4 | 4.2 |
| | 326 | 80.0 ± 3.2 | 4.0 |
| | 3260 | 82.6 ± 2.4 | 2.9 |
| NVP | 50.8 | 100.9 ± 3.2 | 3.2 |
| | 407 | 100.5 ± 2.7 | 2.7 |
| | 4070 | 97.8 ± 1.2 | 1.2 |
| AZT | 200 | 83.6 ± 1.2 | 1.5 |

Matrix effects (%): (peak areas of analytes added post-extraction in six different lots of plasma)/(mean peak areas of standard) × 100.
CV (%): coefficient of variation of matrix effects.

**Table 3.** Extraction recovery for 3TC, d4T, NVP and internal standard in human plasma ($n = 5$)

| Analyte | Analyte concentration (ng/mL) | Extraction recovery (%) | CV (%) |
|---|---|---|---|
| 3TC | 50 | 83.8 ± 6.3 | 7.5 |
| | 400 | 88.0 ± 0.8 | 0.9 |
| | 4000 | 85.2 ± 1.3 | 1.6 |
| d4T | 40.8 | 87.4 ± 5.9 | 6.8 |
| | 326 | 92.3 ± 3.0 | 3.2 |
| | 3260 | 86.1 ± 2.4 | 2.7 |
| NVP | 50.8 | 91.2 ± 6.7 | 7.4 |
| | 407 | 86.3 ± 1.3 | 1.5 |
| | 4070 | 86.6 ± 1.2 | 1.4 |
| AZT | 200 | 86.5 ± 4.7 | 5.4 |

Extraction recovery (%): (peak areas of analytes added pre-extraction in plasma)/(peak areas of analytes added post-extraction in plasma) × 100.
CV (%): coefficient of variation of the extraction recovery in plasma.

suppression on 3TC, d4T and IS. However, the relative matrix effects of these analytes and IS were less than 4.2%, which indicated that there was little variance between different lots of plasma and accurate results could be obtained.

The results of recovery are listed in Table 3. The mean extraction recovery were between 83.8 ± 6.3 and 92.3 ± 3.0% ($n = 5$) for three analytes and IS. The results indicate that the extraction efficiency for all the analytes as well as IS was consistent and reproducible.

### Linearity and Sensitivity

Seven-point calibration curves were prepared ranging from 25 to 5000 ng/mL for 3TC and NVP, and from 20 to 4000 ng/mL for d4T. The curves were obtained by plotting the peak area ratio of the analytes to IS against the corresponding concentration of the analytes in the freshly prepared plasma calibrators. The regression parameters of slope, intercept and correlation coefficient

**Table 4.** Linearity for assay of 3TC, d4T and NVP in human plasma

| Analyte | Run | Linear range (ng/mL) | Calibration curve | *r* |
|---|---|---|---|---|
| 3TC | 1 | 25.0–5000 | $y = 5.67x + 0.027$ | 0.9988 |
| | 2 | | $y = 5.5x + 0.0265$ | 0.9984 |
| | 3 | | $y = 5.76x + 0.0307$ | 0.9987 |
| d4T | 1 | 20.4–4080 | $y = 1.25x + 0.00265$ | 0.9992 |
| | 2 | | $y = 1.24x + 0.000452$ | 0.9991 |
| | 3 | | $y = 1.3x + 0.00243$ | 0.9992 |
| NVP | 1 | 25.4–5080 | $y = -0.217x^2 + 4.78x + 0.00911$ | 0.9992 |
| | 2 | | $y = -0.178x^2 + 4.47x + 0.000206$ | 0.9994 |
| | 3 | | $y = -0.2x^2 + 4.94x + 0.0133$ | 0.9979 |

**Table 5.** Intra-day and inter-day precision and accuracy for assay of 3TC, d4T and NVP in human plasma

| Analyte | AC[a] (ng/mL) | Intra-day ($n = 5$) | | | Inter-day ($n = 15$) | | |
|---|---|---|---|---|---|---|---|
| | | MC[b] (ng/mL) | RSD (%) | A[c] (%) | MC[b] (ng/mL) | RSD (%) | A[c] (%) |
| 3TC | 50 | 53.88 ± 4.0 | 7.4 | 107.8 | 51.69 ± 4.4 | 8.6 | 103.4 |
| | 400 | 428.9 ± 16.0 | 3.7 | 107.2 | 427.1 ± 16.9 | 4.0 | 106.8 |
| | 4000 | 4111 ± 118.6 | 2.9 | 102.8 | 4088 ± 109.3 | 2.7 | 102.2 |
| d4T | 40.8 | 41.51 ± 2.4 | 5.7 | 101.7 | 42.00 ± 2.6 | 6.2 | 102.9 |
| | 326 | 315.6 ± 9.9 | 3.1 | 96.8 | 319.9 ± 10.5 | 3.3 | 98.1 |
| | 3260 | 3359 ± 101.9 | 3.0 | 103.0 | 3341 ± 94.3 | 2.8 | 102.5 |
| NVP | 50.8 | 55.07 ± 1.9 | 3.4 | 108.4 | 54.02 ± 3.5 | 6.5 | 106.3 |
| | 407 | 374.0 ± 13.2 | 3.5 | 91.9 | 389.4 ± 19.8 | 5.1 | 95.7 |
| | 4070 | 4064 ± 183.8 | 4.5 | 99.9 | 4061 ± 146.4 | 3.6 | 99.8 |

[a] AC, analyte concentration. [b]MC, measured concentration. [c]A, accuracy.

**Table 6.** Stability of 3TC, NVP and d4T in human plasma ($n = 3$)

| Analyte | AC[a] (ng/mL) | Three freeze and thaw cycles[b] | −20°C storage for 80 days[b] | Room temperature for 4 h[b] | Extracted samples in autosampler (5°C) for 24 h[b] |
|---|---|---|---|---|---|
| 3TC | 50 | 96.9 | 103.4 | 102.8 | 99.6 |
| | 400 | 100.6 | 103.6 | 107.2 | 107.0 |
| | 4000 | 94.8 | 98.9 | 98.5 | 103.7 |
| d4T | 40.8 | 98.1 | 103.4 | 107.6 | 104.3 |
| | 326 | 94.1 | 100.4 | 101.2 | 100.6 |
| | 3260 | 94.7 | 104.0 | 100.7 | 103.6 |
| NVP | 50.8 | 99.5 | 105.6 | 102.0 | 102.0 |
| | 407 | 96.9 | 100.5 | 102.9 | 102.9 |
| | 4070 | 95.5 | 101.1 | 103.7 | 103.7 |

[a] AC, analyte concentration. [b]Expressed as [(measured concentration)/(analyte concentration) × 100]%.

were calculated by 1/*x*-weighted linear regression for 3TC, d4T and $1/x^2$-weighted quadratic regression for NVP in Analyst 1.4 software used in Sciex API 3000. Excellent linearity was achieved with correlation coefficients greater than 0.9979 for all validation batches. The results are shown in Table 4.

The concentrations of calibration standards were back calculated to obtain the accuracy of each calibration point. Concentrations for QC samples were calculated from the resulting peak area ratios and the regression equation of the calibration curves. The ranges of the calibration points' accuracy for 3TC, d4T and NVP were 86.98–112.2, 86.79–112.4 and 90.74–110.8%, respectively.

The LLOQ of the method is 25 ng/mL for 3TC, NVP and 20 ng/mL for d4T, respectively, which is sensitive enough for our present pharmacokinetic study. The good signal-to-noise (shown in Fig. 2B) obtained at this concentration still allows the LLOQ of the method to be lowered or the volume of plasma used to be decreased.



  

### Precision and Accuracy

The intra-day precision and accuracy were determined by the replicate analyses of QC samples ($n = 5$) at three concentrations. All replicates of the QC samples at each concentration level from three separate validation batches were used to evaluate the inter-day precision. The assay precision and accuracy results are shown in Table 5. The intra-day precision was within 7.4% and the inter-day precision was within 8.6%. The assay accuracy was 91.9–108.4% of the nominal values.

### Stability

The stability of 3TC, d4T and NVP in plasma was investigated and the results are shown in Table 6. They indicate that the analytes were stable after three cycles of freeze and thaw, for 4 h at room temperature and for at least 80 days at −20°C. The stability of processed samples indicated that 3TC, d4T and NVP were stable when kept in the autosampler (5°C) for 24 h.

### Application of the Analytical Method in Pharmacokinetic Study

The study protocol and informed consent forms were reviewed and approved by the Medicine Ethics Committee of Wuhan Union Hospital (Wuhan, China). All subjects provided written informed consent prior to participation in the study.

Twenty-two healthy male volunteers were administered a tablet of lamivudine (300 mg), two capsules of stavudine (15 mg × 2) and a tablet of nevirapine (200 mg) in the fasting state. An indwelling intravenous catheter was inserted following aseptic techniques and blood samples were drawn for the determination of pre-dose concentrations. Pharmacokinetic sampling was performed at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 24, 48, 96, 144, 204 and 240 h. Blood (3.0 mL) was collected in heparinized tubes each time and then centrifuged at 1300***g*** for 10 min. Plasma was separated and stored at −20°C until assay. Figure 3 shows mean plasma concentration–time curves of 3TC, d4T and NVP.

## Discussion

### Development of the Sample Preparing

Customarily, there are three methods for preparing biological specimen, which are liquid–liquid extraction (LLE), solid-phase extraction (SPE) and protein precipitation (PPT). Since, the log*P* of 3TC and d4T is −1.11 and −0.81, respectively, LLE is not suitable for these two NRTIs. Recently, Narang *et al.* (2005) and Mistri *et al.* (2007) described an SPE method. However, the sample preparation process was quite time-consuming and not suitable for high throughput sample analysis.

Compared with other sample preparation techniques, PPT is a simpler and faster method. However, the final extracts are not very clean. In order to eliminate the co-eluting matrix components, and avoid the ion source contamination, a six-port divert valve was used in our method. The eluent was diverted to waste for the first 2.5 min and the last 0.2 min. This process can also effectively cut down the noise caused by these undesirable components. The matrix effects of the method were small and the extraction recoveries of all of the analytes were stable. During the pharmacokinetic study, more than 200 plasma samples could be prepared during a working day, showing high-throughput potential.

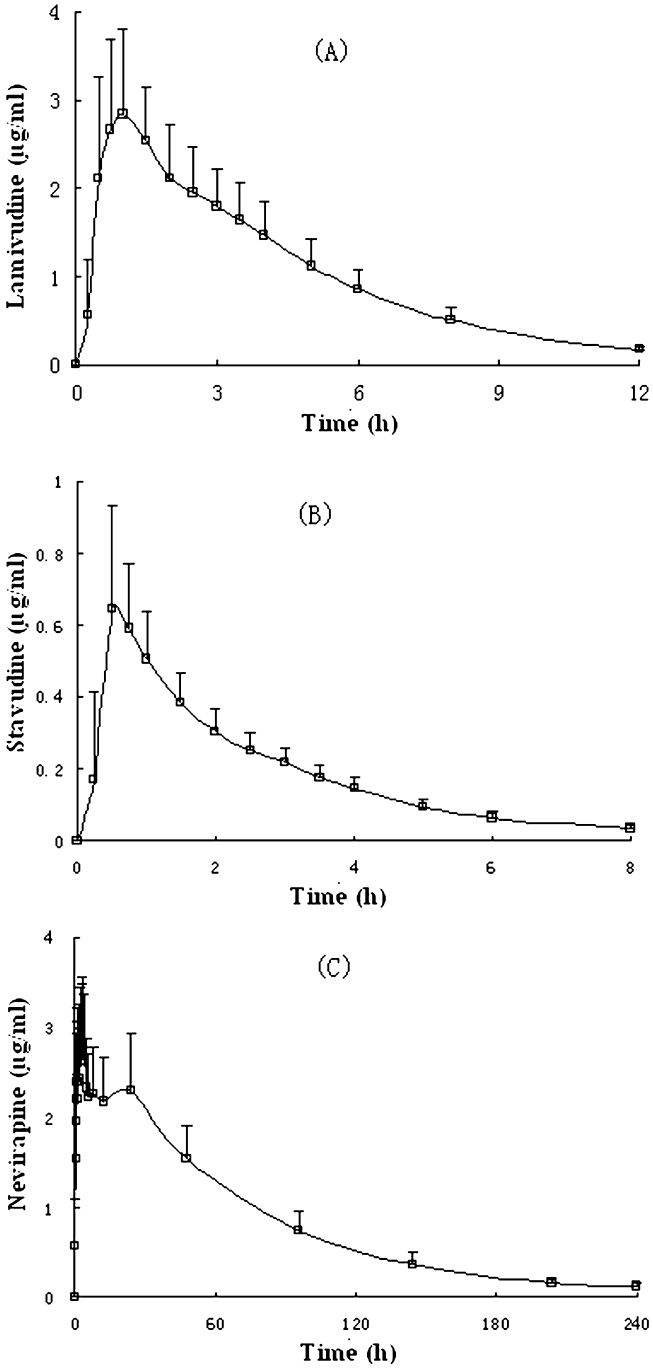


**Figure 3.** Mean plasma concentration–time profiles of (A) lamivudine, (B) stavudine and (C) nevirapine in 22 male healthy volunteers.

### Optimization of Chromatographic Conditions

In this study, it was found that the mass response of these analytes can be stable under acetic acid mobile phase conditions. However, 3TC is a kind of weak base with a $pK_a = 4.3$ (Fridland *et al.*, 2000). In an acid environment, the chromatographic retention of 3TC will be weak, which will lead the 3TC to be vulnerable to matrix effects. Therefore a neutral acetate system was chosen to improve the retention behavior of 3TC and post-column flow injection was used to acidize the eluant.

3TC is hydrophilic compound and NVP is lipophilic compound. To obtain the ideal separation, the ratio of methanol in the mobile phase was adjusted through the gradient profile. The low ratio of methanol can avoid 3TC being eluted early with other polar interfering substances and the high ratio of methanol can shorten the total analysis time.

### The Choice of Internal Standard

Zidovudine is also a kind of NRTI, and has the similar structure and mass spectra behavior to 3TC and d4T. It can also compensate for the deviation caused by matrix effects. The accuracy and precision of the method have shown that zidovudine is a suitable internal standard.

## Conclusion

A fast, sensitive and specific LC-MS/MS method for simultaneous determination of lamivudine, stavudine and nevirapine in human plasma was developed and validated. To the best of our knowledge, this is the first report describing protein precipitation as a sample preparation method for determining these three analytes together. Compared with the published methods, the steps of sample preparation were very simple and the volume of plasma used for sample preparing was reduced dramatically. By using 150 μL plasma samples, the lower limits of quantification were achieved. The method has been successfully applied to the clinical pharmacokinetic study and satisfactory results have been obtained. It demonstrates that the method is reproducible and suitable for high-throughput sample analysis.

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