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An LC-MS-MS method for the determination of nevirapine, a non-nucleoside reverse transcriptase inhibitor, in human plasma

Jingduan Chi [a,b,*], Anura L. Jayewardene [a], Judith A. Stone [a], Francesca T. Aweeka [a]

[a] *Drug Research Unit, Department of Clinical Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143, USA*
[b] *Anti-infective Research Laboratories, Department of Clinical Sciences and Administration, College of Pharmacy, Texas Medical Center, University of Houston, 1441 Moursand Street, Houston, TX 77030, USA*

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Abstract

A sensitive and rapid liquid chromatography tandem mass spectrometry (LC-MS-MS) method has been developed to measure the levels of the HIV-1 non-nucleoside reverse transcriptase inhibitor nevirapine (NVP) in human plasma. The analyte and internal standard (IS) are isolated from plasma by a simple perchloric acid precipitation of plasma proteins followed by centrifugation. LC-MS-MS in positive mode used pairs of ions at *m/z* of 267/226 for NVP and 628/421 for the IS, respectively. Two linear calibration curves were established for quantitation of NVP with the low curve ranging from 25 to 1000 ng/ml and the high curve ranging from 1000 to 10 000 ng/ml. Mean inter- and intra-assay coefficients of variation (CVs) over the ranges of the two standard curves were less than 10%. The overall recovery of NVP was 92.4%.

Keywords: Nevirapine; LC-MS-MS system; Plasma

1. Introduction

Nevirapine (NVP), 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido-[3,2-b:2′,3′-e] [1,4] diazepin-6-one (Fig. 1), a non-nucleoside reverse transcriptase inhibitors (NNRTI) is a noncompetitive inhibitor of reverse transcriptase, an important therapeutic target for treatment of HIV-1 [1]. Several HPLC-UV methods for quantitation of NVP in plasma have been recently published [2–4]. However, these methods use solid phase extraction or plasma protein precipitation followed by evaporation of extracts to obtain limits of quantitation ranging from 25 to 52 ng/ml. Presently no published method using LC-MS-MS for quantitation of NVP in plasma is available. The method reported in this paper uses protein precipitation and LC-MS-MS to quantitate NVP in plasma. The method is rapid, both in sample preparation and analysis. Only 50 ul of sample is required which is

* Corresponding author. Tel.: +1-713-795-8319; fax: +1-713-795-8383.
E-mail address: jchi@mail.uh.edu (J. Chi).

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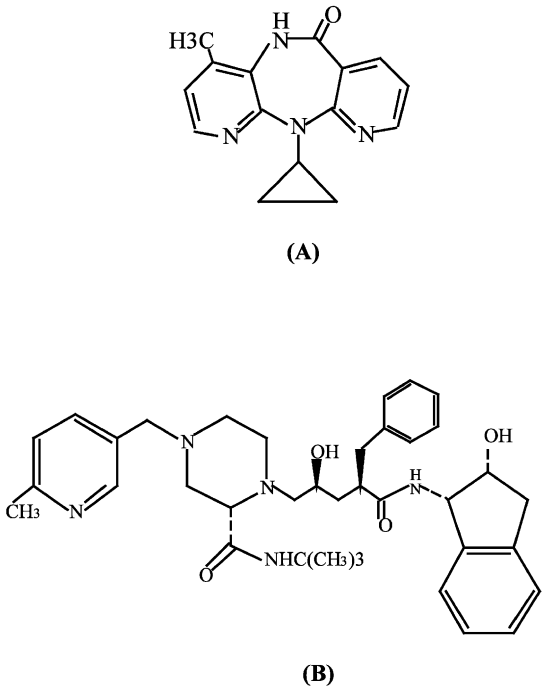

Fig. 1. Chemical structures of NVP (1) and IS (2).

advantageous for use with pediatric samples. Furthermore, the absence of interference during MS-MS quantitation permits use of a small LC column enabling rapid analysis. A further advantage of this method is the ability to analyze additional drugs, such as nelfinavir and indinavir, concurrently without changing the column or mobile phase. Therefore, the method and validation of this rapid LC-MS-MS assay for NVP in plasma using an internal standard (IS) is described.

2. Experimental

2.1. Materials

All chemicals were HPLC grade or reagent grade unless otherwise noted. Acetonitrile ammonium formate, formic acid (99–100%), perchloric acid (69–72%), ammonium hydroxide (25% in water) and HPLC-water were from Fisher Scientific (Fair Lawn, NJ, USA). NVP was obtained from Boehringer–Ingelheim Pharmaceuticals (Ridgefield, CT, USA). Methylindinavir sulfate (IS) was supplied by Merck Research Laboratory (Rahway, NJ, USA). Drug free human plasma was obtained from the Long Hospital Blood Bank (University of California at San Francisco, San Francisco, CA, USA). Snap cap micro centrifuge tubes (1.5 ml) from Fisher Scientific.

2.2. Apparatus

The PE Sciex API 2000 triple quadrupole mass spectrometer with Turboion spray sample inlet was purchased from Perkin–Elmer–Sciex (Concord, Ont., Canada). Perkin–Elmer, Norwalk, CT supplied the PE Biosystems 200 series autosampler and twin PE Biosystems series 200 micro HPLC pumps.

2.3. MS-MS parameters

Mass spectral analyses was accomplished on a Model API 2000 triple quadrupole mass spectrometer, fitted with an API Turboion spray source and operated in the positive ionization mode. Nebulizer, turbo and curtain gases used ultra high purity nitrogen (99.999%) delivered at settings of 45, 55 and 30 psi, respectively. Nitrogen was also used as the collision gas at a setting of 4, which produced an overall analyzer pressure of approximately 2.6×10^{-5} Torr. The Turboion spray temperature was maintained at 495 °C. The collision energy was −47 V. Tuning parameters were optimized in order to get the best signal to noise (S/N) ratio for NVP. The mass spectrometer was operated in MS-MS mode using multiple reaction monitoring (MRM) to detect specific precursor ion to product ion transitions for each analyte. Following chromatographic separation, the protonated ion ($M+H^+$) for each analyte was selected by the first quadrupole (Q1) and focused into the collision cell (Q2) where it fragmented into product ions. For each $M+H^+$ precursor ion, a distinct product ion was selected by the third quadrupole (Q3). The precursor ion intensity was monitored and subsequently stored by the computer system of the mass spectrometer. These mass spectral Q1/Q3 transitions, monitored

for NVP and IS, were 267/226 and 628/421 (m/z), respectively. A 5 ms delay between scans was found to be adequate for eliminating potential cross talk. The control software, including LC TUNE, MULTIVIEW, TURBOQUAN, METHOD EDITOR and EXPERIMENT EDITOR was installed on a Macintosh OS 8.5 platform with 64 MB of RAM and a 16 GB hard disk.

Instrument tuning parameters were optimized using 1000 ng/ml solutions of NVP and IS prepared in acetonitrile–ammonium formate buffer (pH 4, 10 mM) (50:50, v/v). These solutions were infused separately at 10 μl/min into the LC-MS-MS interface using a built-in syringe pump.

2.4. Liquid chromatography parameters

The specific liquid chromatographic parameters for the assay were as follows: The column used was a Zorbax XDB-C8 reverse phase column, 2.1 mm i.d. × 50 mm, with 5 μm particle size packing and a Zorbax–Eclipse polymeric 2.1 × 12.5 mm guard column. The flow rate was kept constant at 400 μl/min. Mobile phase A was a buffer and mobile phase B was acetonitrile. The flow gradient was initially 80:20 v/v of A:B for 0.20 min, linearly ramped to 20:80 over 0.6 min, held at 20:80 for 2.4 min, and then returned to 80:20 over 0.3 min. This condition was held for a further 1.5 min prior to the injection of another sample. The volume of injection was 20 μl through a 100 μl loop.

Mobile phase A was prepared by dissolving 1.28 ammonium formate in 2.0 l HPLC-grade water and adjusting the pH to 4.1 with formic acid prior to filtration through a 0.2 μm membrane. This was followed by sonication under vacuum to degas. The mobile phase B was a 0.1% v/v solution of pure formic in HPLC-grade acetonitrile which was mixed well, filtered and degassed under vacuum.

2.5. Preparation of standards and controls

To prepare NVP stock solution, 5.0 mg NVP was accurately weighed and dissolved in a 5 ml volumetric flask with 60% acetonitrile in water to generate a concentration of 1000 μg/ml of NVP. This was diluted appropriately to lower concentrations for spiking the calibration standards. These were prepared by spiking 5 ml aliquots of drug-free plasma with the NVP working standard solutions to give a range of concentrations from 25.0 to 10 000 ng/ml. Frozen quality control (QC) pools were prepared at four different concentrations of NVP using a separate stock solution of NVP containing 1000 μg/ml in aqueous acetonitrile. The IS (methyl indinavir) was prepared in aqueous acetonitrile (50:50 v/v) at a concentration of 100 μg/ml and further diluted 1:100 v/v to a working concentration of 1.0 μg/ml with aqueous acetonitrile (75:25 v/v). Aliquots (50 μl) of calibration standards and QC samples were pipetted into 1.5 ml polypropylene snap cap tubes and stored frozen at −20 °C until required for analysis.

2.6. Processing of plasma samples

Frozen plasma samples from study subjects, QC samples and calibration standards were thawed as needed. The same procedure was followed for all samples. One hundred microliters of IS solution was added to aliquots of 50 μl of plasma from deactivated study samples, calibrators and QC in 1.5 ml microcentrifuge tubes, and mixed gently. Two hundred microliters 1.5 M perchloric acid was added to each tube and vortexed for 20 s at high speed. The tube was centrifuged at 12 000 × g (11 000 rpm) for 5 min to pellet the precipitated proteins and give a clear supernatant. Two hundred microliters supernatant was taken and neutralized with 200 μl of 2% ammonium hydroxide in 40% acetonitrile. These neutralized extracts were transferred to vial inserts and placed in the autosampler tray for injection onto the LC column.

2.7. Data analysis

Calibration standards in plasma containing 25, 50, 75, 250, 500, 1000, 2500, 5000, 7500 and 10 000 ng/ml were used to establish two calibration curves with $1/x$ weighted linear regression. NVP concentrations versus peak area ratios were plotted in TURBOQUAN, a separate quantitation software program supplied with the mass spectrometer.

3. Results

3.1. Linearity

Extracted ion chromatograms from a patient sample with NVP and added IS and drug-free plasma blank are shown in Fig. 2. Calibration standard in human plasma containing 25 to 10 000 ng/ml of NVP were used to establish two calibration curves for assay validation. Linear regression of concentration versus peak area ratio plots of two curves resulted in coefficients of determination (r^2) consistently greater than 0.995. The reproducibility of the calibration standards over 6 days of assay are indicated by regression parameters, with mean slopes of 0.00095 ± 0.00226 for low standard curves, 0.00066 ± 0.33491 for high standard curves. The mean coefficients of determination were 0.9988 ± 0.0007 with a CV of 0.07% for the low standard curve, and 0.9972 ± 0.0015 with a CV of 0.15% for the high standard curve over 6 days.

3.2. Precision and accuracy

Inter- and intra-assay precision was evaluated at four concentrations of 100, 300, 1500 and 6000 ng/ml designated as low, medium, high and extra high. For inter-assay precision, six samples of each concentration were assayed on 6 different days using six sets of standard curves. Means and standard deviations (S.D.) were obtained for the calculated drug concentrations over all 6 days and coefficients of variation (CV%) for the four different levels ($n = 36$ for each) were determined. For intra-assay precision, ten control samples from each of four concentrations were assayed with a single calibration curve and CV for the calculated analyte concentrations were determined. As shown in Table 1, intra-assay precision ($n = 10$) ranged from 2.3 to 6.4% and the accuracy was within 92.2–101.1% of the nominal concentration of the QC samples. Inter-assay precision ($n = 6$) ranged from 4.0 to 8.0% and the accuracy

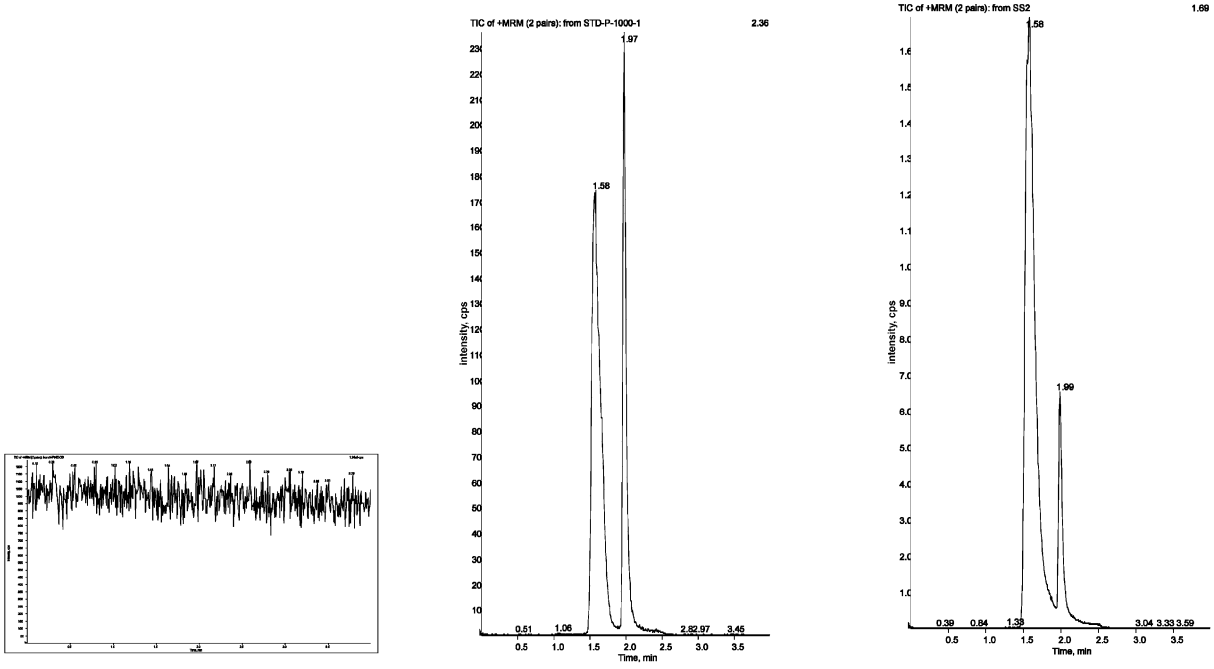

Fig. 2. Chromatograms of (A) blank plasma, (B) drug-free plasma spiked with 1000 ng/ml NVP and 142 ng/ml of the IS and (C) Chromatogram of a sample from a patient following administration of NVP which its calculated concentration of NVP was 4757 ng/ml. Peaks 1 and 2 represent NVP and the IS, respectively. The injection volume was generally 20 μl on column.

Table 1
Intra- and inter-assay precision (R.S.D.s) and accuracy data for the determination of NVP in plasma

Actual value (ng/ml)	Observed value (ng/ml)		CV (%)	
	Intra	Inter	Intra	Inter
100	92.2±5.9	101.8±6.2	6.4	6.1
300	297.9±6.8	298.8±12.1	2.3	4.0
1500	1516.4±73.4	1520.5±78.9	4.8	5.3
6000	5827.3±213.2	6111.4±258.4	3.7	8.0

The intra- and inter-assay data represent the mean±S.D. of ten and 36 observation, respectively.

was within 99.6–101.9% of the nominal concentration of the QC samples.

3.3. Limit of quantitation and recovery of nevirapine

The intra-assay lower limits of quantitation were determined by adding NVP to ten aliquots of blank plasma at a concentration of 25 ng/ml and assaying them with a set of calibration standards. The mean, S.D. and the CV% were determined. The lower limit of quantitation for NVP by this method was 25 ng/ml with a CV of 6.8% (ten replicates) and a mean accuracy of 103.6%. For the inter-assay limit of quantitation over 6 days, the CV was 2.3% and the accuracy was 105.6%.

Recovery of NVP from plasma following sample preparation was assessed by comparing the concentration of analyte from plasma samples to the concentration of drug spiked into the mobile phase at the same concentration as in the plasma samples. In order to avoid the loss of IS during sample preparation, the IS was added after plasma samples were precipitated and centrifuged. Overall mean recovery of NVP was found to be 92.4% over concentration ranges of 25–10 000 ng/ml.

3.4. Stability

3.4.1. Freeze–thaw stability

Stability of NVP in human plasma during repeated three freeze–thaw cycles was evaluated for 100, 300, 1500 and 6000 ng/ml samples. The calculated concentrations after repeated freezing and storage at −20 °C were compared with the calculated concentrations of freshly spiked plasma at same concentrations. The data presented in Table 2 shows that the deviation from nominal did not exceed 10% for any of the NVP concentrations.

3.4.2. Stability at room temperature

Frozen NVP controls in plasma at the four concentrations were thawed and left on the bench at room temperature for 24 h. These samples, a set of freshly thawed samples, and a set of calibration standards were analyzed and the NVP concentrations of each were compared with determine the stability of the NVP during storage at room temperature. NVP was stable at room temperature for 24 h with no change observed.

3.4.3. Stability of extracted nevirapine at room temperature

A set of six aliquots of frozen NVP controls in plasma at 100, 300, 1500 and 6000 ng/ml were thawed and extracted following the sample preparation procedure, and then left on the bench at room temperature for 24 h. These samples, a set of freshly thawed samples, and a set of calibration standards were analyzed and the NVP concentrations of each were compared with determine the stability of the extracted NVP during storage at room temperature. Extracted NVP was stable at room temperature for 24 h with no change observed.

3.5. Application

This method has been used for the measurement of NVP concentrations in patient plasma. Fig. 3

Table 2
Result of NVP freeze–thaw stability

Freeze–thaw # cycles[a]	QC-[100 ng/ml] mean calculated concentration	QC-[300ng/ml] mean calculated concentration	QC-[1500ng/ml] mean calculated concentration	QC-[6000ng/ml] mean calculated concentration
1	104.5	315.2	1400	5738
2	98.5	318.3	1365	5763.8
3	98	338.6	1539	6033
Changes from cycle 1 to 3 (%)	−6.2	7.4	9.9	5.1

[a] $n = 6$ samples for each cycle freeze–thaw.

shows a patient plasma profile of NVP following multiple oral dose administration of 400 mg of NVP.

4. Discussion

Quantitative analysis with high selectivity and sensitivity by LC-MS-MS uses the technique of selected MRM. In this mode only a selected MS-MS or collision induced dissociation (CID) transition needs to be monitored. This is now considered the best analytical approach for accurate and highly selective quantitative measurement of drugs and metabolites in complex matrices such as biological samples. In this method the unique pair of ions with m/z 267 and 226 for NVP and m/z 628.4 and 421.2 for the IS, respectively, constitute the parent/daughter ion pairs which are scanned alternatively every 200 ms with 5 ms

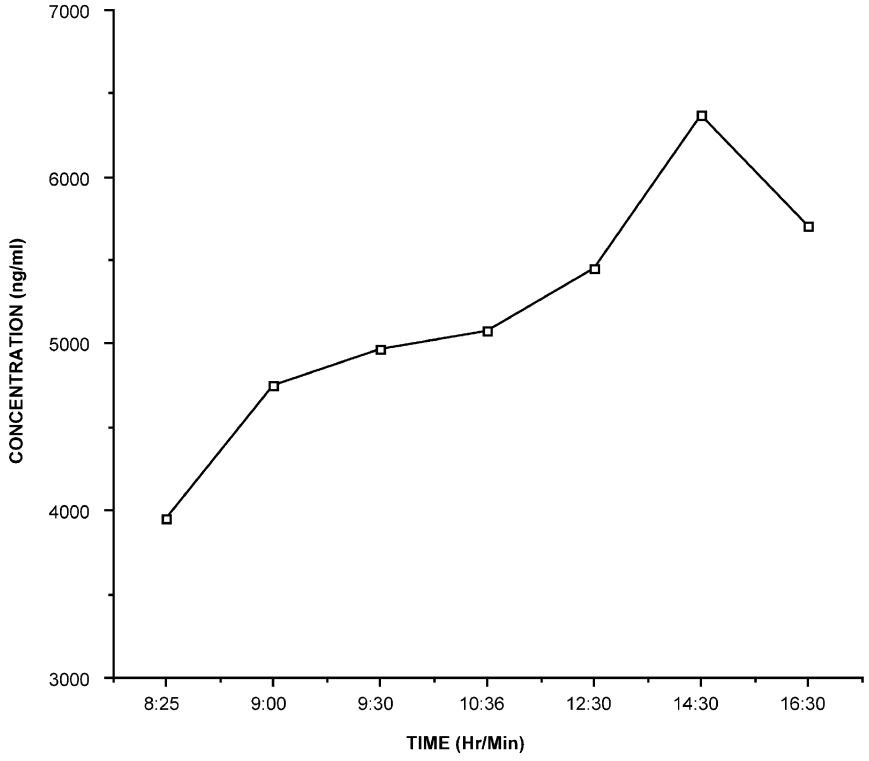

Fig. 3. Patient plasma concentration–time profile obtained following multiple dose administration of 400 mg NVP.

delay between scans. There was no interference observed from residual endogenous plasma components and from other concomitantly administered drug analytes. The absence of interference from other analytes was clearly seen in the analysis of NVP buffer sample, which was spiked with other antiretroviral agents including zidovudine, indinavir, ritonavir, saquinavir, nelfinavir and stavudine.

Two linear calibration curves were established for quantitation of NVP with the low curve ranging from 25 to1000 ng/ml and the high curve ranging from 1000 to10 000 ng/ml. It was not possible to obtain a single linear curve with the wide range of 25–10 000 ng/ml using the LC-MS-MS method, and therefore, results were generated using two calibration curves as shown in Table 1. The broad calibration curve range of 25.0–10 000 ng/ml was adequate for the handling of most pharmacokinetic samples.

5. Conclusion

Among the significant advantages of this method are high selectivity, accuracy, recovery and the low limit of quantitation for NVP. Furthermore, the rapid and simple sample preparation associated with the method, the short run time of 5 min and the need for only 50 μl of plasma, as compared with 150–250 μl necessary for HPLC methods, are added advantages. This method has been successfully used to determine NVP concentrations within plasma samples obtained from patients.

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References

[1] R.L. Murphy, J. Montaner, Expert Opinion on Investigational Drugs 5 (1996) 1183–1887.
[2] R.M.F. Hollanders, D.M. Burger, Journal of Chromatography B 744 (2000) 65–71.
[3] R.P.G. van Heeswijk, R. Hoetelmans, J.H. Beijnen, Journal of Chromatography B 713 (1998) 395–399.
[4] J.W. Pav, L. Rowland, D.J. Korpalski, Journal of Pharmaceutical and Biomedical Analysis 20 (1999) 91–98.