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A Sensitive and Specific Liquid Chromatography/Tandem Mass Spectrometry Method for Quantification of Nevirapine and Its Five Metabolites and Their Pharmacokinetics in Baboons

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Abstract

A highly sensitive and specific LC-MS/MS assay was developed and validated to quantify nevirapine (NVP) and its five metabolites (2-, 3-, 8-, 12-hydroxyl NVP [OHNVP] and 4-carboxyl NVP [CANVP]) simultaneously in baboon serum and the assay was used to characterize their pharmacokinetic studies of an oral-dose escalation study in baboon. The lower limit of quantification (LLOQ) for NVP and its four hydroxyl nevirapine metabolites is 1.0 ng/mL, at least 10-fold higher than reported methods. The LLOQ for 4-CANVP is 5.0 ng/mL. The between-run and within-run precisions and accuracies at the following quality control concentrations (1, 5, 50 and 500 ng/mL) were evaluated in baboon serum with less than 14% variation and 93% to 114% accuracies (n=6) except LLOQ for 2-OHNVP, which had an accuracy of 115.8% for between-run validation. The pharmacokinetics of NVP and its five metabolites in non-pregnant baboons by single dose escalation study were also profiled. The major metabolites detected were 4-CANVP and 12-OHNVP. 3-OHNVP and 2-OHNVP were the minor metabolites with only a trace amount of 2-OHNVP detected in some PK samples. No 8-OHNVP was observed in all of the PK samples. In addition, the fragmentation for the four hydroxyl metabolite isomers was also discussed.

Keywords

nevirapine; metabolites; quantification; pharmacokinetics

Introduction

Nevirapine (NVP), a dipyrindodiazepinone, inhibits the replication of the human immunodeficiency virus-1 (HIV-1) by binding directly to the catalytic site of reverse transcriptase and disrupting the RNA- and DNA- dependent DNA polymerase activities. (Merluzzi et al., 1990; Richman et al., 1991; Murphy and Montaner, 1996) Being the first non-nucleoside reverse transcriptase inhibitor, NVP has a long half-life and is readily absorbed, with a bioavailability exceeding 90% in humans following oral administration in

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tablet or liquid formulation.(Lamson et al., 1999) Nevirapine undergoes an extensive cytochrome P450 oxidative metabolism with subsequent glucuronide (ether) conjugation of hydroxylated metabolites and excretion in the urine and feces.(Riska et al., 1999a; Riska et al., 1999b) The biotransformation of NVP to the four hydroxyl metabolites (2-, 3-, 8- and 12-hydroxyl NVP) are primarily formed through the mediation of CYP3A and CYP2B6, and to a less extent, CYP2D6 and CYP2C9.(Riska et al., 1999a) Another metabolite, 4-carboxylic nevirapine (4-CANVP), is formed through the secondary oxidation of 12-OHNVP.(Riska et al., 1999b)

Nevirapine-based regimens using two nucleoside reverse transcriptase inhibitors (NRTIs) have been shown to effectively suppress HIV-1 replication in antiretroviral-naïve patients. (D'Aquila et al., 1996; Montaner et al., 1998; van Leeuwen et al., 2003) In resource-limited countries, a single-dose NVP regimen administered to pregnant mothers at the onset of labor and newborns immediately after birth significantly reduced perinatal HIV-1 transmission rates among breastfeeding populations.(Guay et al., 1999) Recently, several studies and case reports have shown increased incidence of NVP-associated hepatotoxicity among pregnant and non-pregnant women with CD4+ cell counts > 250/mm³, and men > 400/mm³(Merluzzi et al., 1990; Knudtson et al., 2003; Stern et al., 2003; Hitti et al., 2004; Joy et al., 2005). However, symptomatic hepatotoxicity has not been reported with the use of a single-dose NVP for prevention of perinatal HIV-1 infection. Recently, the clinical use of NVP-based regimens has been discouraged unless the benefits outweigh the risks.

There is increasing evidence that virologic treatment responses and adverse events of NNRTIs is, at least in part, due to the significant variations in plasma drug levels among different individuals. One of the factors that have been intensively studied is the association between the cytochrome 2B6-516G>T polymorphism and drug exposure of NVP and efavirenz (EFV), two of the three currently available NNRTIs.(Haas et al., 2004; Rotger et al., 2005; Penzak et al., 2007) Individuals with the CYP 2B6 G516T substitution have significantly reduced function of this isoenzyme, and are characterized as a “slow metabolizer” phenotype.(Rotger et al., 2005) In a recent study, a 1.7-fold higher NVP plasma level was reported among CYP 2B6 516TT homozygotes. In addition, greater plasma and intracellular exposure and central nervous system adverse effects to EFV was reported among patients with CYP 2B6 516 TT genotype.(Rotger et al., 2005)

The association between CYP 2B6 polymorphism and NVP-associated hepatotoxicity remains unknown. Pharmacokinetic studies are needed to verify drug enzyme metabolism genetic variants and the metabolite profiles of NVP. Therefore, it is very important to monitor the parent drug and its five metabolites simultaneously in order to better understand the fate of the drug and verify the toxicological species. Although several methods (van Heeswijk et al., 1998; Pav et al., 1999; Hollanders et al., 2000; Silverthorn and Parsons, 2006) were reported to quantify NVP in biological matrices with the lowest detected concentration of 25 or 10 ng/mL using UV or mass spectrometric detection coupled with liquid chromatographic separation, respectively. Till recently, a rapid highly sensitive LC-MS/MS method to quantify NVP and three of its metabolites (2- and 3-OHNVP as a composite, and 4-CANVP) in baboon serum (Liu et al., 2007) were established to determine the dose required to achieve comparable plasma level of NVP in a single dose-escalation study. The other metabolites were not quantified due to a lack of authentic samples of 3-OHNVP, 8-OHNVP and 12-OHNVP at the time of method development. Since we obtained 3, 8, 12-OHNVP pure compounds (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CO), we adapted this method for simultaneously quantification of NVP and five metabolites in both baboon. During this course, a rapid LC-MS/MS method to quantify five oxidative metabolites of nevirapine in human plasma(Rowland et al., 2007) was reported. Here, we reported a highly sensitive and specific LC-MS/MS method capable of quantifying NVP and

five oxidative metabolites simultaneously and its application to profile NVP and its five metabolites in baboons. In addition, the mass spectral fragmentation of NVP and metabolites was also proposed.

Experimental

Materials

All NVP standards and formulations, including NVP oral solution at 10 mg/mL and 200 mg tablets, the pure powder of NVP and its five metabolites (2-OHNVP, 3-OHNVP, 8-OHNVP, 12-OHNVP and 4-CANVP), were obtained from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CO). The internal standard, hesperetin, was provided by the National Cancer Institute (NCI). An E-pure water purification system (Barnstead, Dubuque, IA) was used to obtain the HPLC grade water (>18 mΩ). All organic solvents were of HPLC grade and were obtained from Fisher Scientific (Pittsburg, PA). Formic acid (FA) was of reagent grade and was purchased from Sigma (St. Louis, MO). All chemicals and reagents were used as received.

Liquid Chromatographic and Mass Spectrometric Operating Conditions

The LC-MS system used consisted of a Finnigan TSQ Quantum EMR Triple Quadrupole mass spectrometer (Thermo Fisher Scientific Corporation, San Jose, CA) coupled to Shimadzu HPLC system (Shimadzu, Columbia, MD), which was equipped with a CBM-20A system controller, an LC-20 AD pump, a SIL-20AC autosampler, CTO-20A column oven, DGU-20A5 degasser and FCV-11AL valve unit. The temperature of the auto-sampler was set at 4°C during operation. Nevirapine, its five metabolites and the internal standard hesperetin were separated on an Aquasil C₁₈ column (2.1×50 mm, 5 μm, Thermo Hypersil-Keystone, Bellefonte, PA) coupled with an Aquasil C₁₈ guard column (2.1×10 mm, 2 μm, Thermo Hypersil-Keystone, Bellefonte, PA) (a drop-in guard cartridge with a uniguard holder) under a gradient elution at a flow rate of 0.20 mL/min. The mobile phase A consisted of water/0.1% formic acid and the mobile phase B consisted of acetonitrile (ACN) /0.1% formic acid. The gradient initiated from 5% B, increased to 10% B in 1 min. After holding at 10% for 18 min, the gradient increased to 50% B in 2 min and the column was washed for another 2 min before returning to 5% B in 1 min. The column was equilibrated at 5% B for 6 min before the next sample injection. The total run time was 30 min. In the first 8 min, the eluent was diverted to the waste.

The mass spectrometer was operated in the positive ESI mode with a helium pressure of 20 psi, a typical electrospray needle voltage of 4700 V, a sheath nitrogen gas flow of 49 (arbitrary unit) and a heated capillary temperature of 300°C. The samples and the internal standards were analyzed by multiple reaction monitor (MRM) mode using ion transitions at m/z as follows: NVP: 267.01>226.01 (E=30%); 2-OHNVP: 283.00>161.11 (E=26%); 3-OHNVP: 283.01>214.00 (E=30%); 4-CANVP: 297.07>209.84 (E=30%); 8-OHNVP: 283.05>241.90 (E=27%); 12-OHNVP: 283.03>265.00 (E=20%); Hesperetin: 303.10>176.96 (E=19%). The parent mass of the four hydroxyl metabolite isomers were set to be slightly different in order to be better differentiated by multiple reaction monitoring mode. The mass spectrometer was tuned to its optimal sensitivity by direct infusion of 4-CANVP, since it had the lowest sensitivity. The effect of collision energy on the product ion intensities of the four hydroxyl metabolites were also investigated by direct infusion of their 10 μg/mL 50% ACN solution containing 0.1% formic acid. All operations were controlled by Finnigan Xcalibur software on a Windows NT 4.0 system.

The high resolution mass measurements of the parent ions and product ions of NVP and its five metabolites were performed on LTQ-Orbitrap (Thermo Fisher Scientific Corporation, San Jose, CA) mass spectrometer equipped with nanospray source operated in positive ion

mode. Samples (10 µg/mL) prepared in 50% ACN/0.1% FA were infused into the nanospray source at a rate of 0.5 – 1 mL/min. The optimized conditions were as follows: spray voltage 3 KV, capillary temperature 230°C, capillary voltage of 9 V and a tube lens voltage of 100 V. The resolution on the Orbitrap was set to 30,000 and the scan range was m/z 100 – 2000. Ten scans were acquired in profile mode and averaged. Parent ions were manually isolated. The collision induced dissociation fragmentation energy was between 20 – 25% and set at a value optimal for the compound of interest.

Sample Preparation

Ten µL of the internal standard hesperetin solution (10 µg/mL stock in 50% ACN) was added into a 0.1 mL of baboon serum sample. The above mixture was then extracted with 1.0 mL ethyl acetate by vortex mixing for 1 min at room temperature. After centrifuging at 14,000 g for 4 min, the organic layer was transferred to a clean borosilicate glass tube and evaporated to dryness under a mild stream of nitrogen. The residue was reconstituted in 100 µL of 5% ACN/0.1% FA and a 50 µL aliquot was injected into the instrument for analysis.

Assay Validation

Mixtures of NVP and its metabolites in the concentration range of 1–1000 ng/mL were spiked into 0.1 mL baboon serum with a constant amount hesperetin (1000 ng/mL) to make serum samples for standard curves. The within-run precision values were determined in six replicates at concentrations of 1, 5, 50 and 500 ng/mL. The between run precision was determined also across these concentrations in six replicates. The mean concentration and the coefficient of variation (CVs) were calculated. The accuracy of the assay was determined by comparing the nominal concentration with the corresponding calculated mean concentration.

Pharmacokinetic Study

The procedures for the oral administration of NVP to baboons and its single-dose escalation PK studies were reported previously (Liu et al., 2007). Briefly, eight non-pregnant female olive baboons (*Papio Anubis*) received an oral administration of NVP (oral solution or pulverized powder mixed with banana and bread mesh) at different doses. At each time point (0, 1.5, 4 and 8 hrs), a 2.0 mL blood sample was collected. The serum samples were then separated from the blood by centrifugation and stored at –80°C until analysis.

Noncompartmental pharmacokinetic analysis was performed to obtain the relevant PK parameters of NVP and its metabolites by the WinNonlin computer software, version 5.0 (Pharsight Corporation, Mountain View, CA).

Result and Discussion

Fragmentation and Chromatographic Separation of NVP and Its Metabolites

The 1 min average mass spectra of NVP, its four hydroxylated metabolites (2-, 3-, 8- and 12-OHNVP) and 4-CANVP on a TSQ Quantum Ultra AM mass spectrometer under ESI positive mode showed the following predominant ions at m/z 267.0, 283.0, 297.1, corresponding to their protonated molecular ions $[MH]^+$, respectively, similar to what we have reported before (Liu et al., 2007). These protonated molecular ions were selected for fragmentation by collision induced dissociation (CID) and their tandem mass spectra are shown in Figure 1. Similar to fragments of the $[MH]^+$ of NVP (m/z 267.0) reported before, a single peak at m/z 226.0 was observed in its CID spectrum (Figure 1A). Similar fragment peaks at m/z 161.1, 214.0 and 242.0 are shown in tandem mass spectra of both 2-OHNVP (Figure 1B) and 3-OHNVP (Figure 1C), suggesting that the fragmentation pathways of 2-OHNVP and 3-OHNVP are similar under this condition. In 8-OHNVP, the peak at m/z

242.0 was predominant with a low abundance peak at m/z 177.1 (Figure 1E). A one single peak at m/z 265.0 was observed in the CID spectrum of 12-OHNVP (Figure 1F). In order to understand the molecular mechanism of their fragmentation, various collision energies were evaluated. The relative ion intensity ratios of the parent ions (m/z 283.1) and the three product ions (m/z 161.1, 214.1 and 242.1) to the total ionic intensity were plotted as shown in Figure 2. With increasing collision energy from 10% to 35%, the relative ratio of the parent ions (m/z 283.1) of 2-, 3- and 8-OHNVPs fell rapidly and no parent ions was detected at 35% collision energy. For 2-OHNVP, the relative ratios of the product ions at m/z 161.1 and 214.1 are larger than that of the product ion at m/z 242.1 with >25% collision energy (Figure 2A). The product ion at m/z 214.1 in 3-OHNVP was predominant over the presence of the other two product ions at m/z 161.1 and 242.1 with >20% collision energy (Figure 2B). However for 8-OHNVP, the product ion at m/z 242.1 was the most predominant ion with a negligible abundance of the other two fragment ions (Figure 2C). In spite of varying collision energy from 20% to 30% only a single peak at m/z 265.0 was observed in its tandem mass spectrum of 12-OHNVP. This peak is putatively assigned as the dehydrated ion of the protonated 12-OHNVP. The ionic structure may be a very stable N-analog tropylium-like cation, which was labeled as fragment 1B (F1B) in Figure 3A. It may be formed through the rearrangement of an initially formed pyridyl-4-methylene cation (F1A) by dehydration of the protonated 12-OHNVP ion.

Based on the above collision energy-dependent fragmentation of these protonated molecular ions of 2-, 3- and 8-OHNVPs, a concise fragmentation pathway of 2-, 3- and 8-OHNVPs were tentatively proposed in Figures 3B–3E. As depicted in Figure 3B, 2- and 3-OHNVPs may readily lose the cyclopropyl radical to yield a delocalized stable conjugated radical cation at m/z 242.1 (labeled as F2). The fragmentation pathway is also applicable to the protonated molecular ion of NVP evidenced as a sole fragment ion of 226.01 in the tandem mass spectrum of NVP at 30% collision energy (Figure 1A). The relative intensity of the three radical cations as shown in Figure 2 demonstrated that the stability order of these radical cations are 8-OHNVP>3-OHNVP>2-OHNVP. Their relative stability may be attributed to the lone electron pair of oxygen and lone electron pair of nitrogen repulsion in the 2-OHNVP and the potential steric repulsion of 3-OH and 4-methyl group in 3-OHNVP. These radical cations can further fragment to other fragment ions at m/z 214 or 161, which is collision energy and structural dependent. Loss of a carbon monoxide moiety from the radical cation gives a 9,10-dihydro-1,8,9,10-tetraaza-anthracene radical cation with m/z 214.1 (labeled as F3) (Figure 3B) and their intensity seems associated with their precursor radical cation stability. It is a common fragmentation pathways observed in benzodiazepines compounds since it generates a resonance stabilized six-membered ring from the seven-membered ring. (Smyth et al., 2000) Alternatively, the protonation of the molecular ion may also take place on the carbonyl oxygen (Figure 3C). The direct result of this protonation is through the attack with the lone pair of electrons on the 11th nitrogen. After the addition-elimination mechanism, a neutral bicyclic molecule was expelled resulting in the formation of the product ion at m/z 161.1 (labeled as F4A). This structure is very similar to N-alkylbenzoazetiones, which has already been synthesized by Olofson group. (Olofson RA, 1984a; Olofson RA, 1984b) It can also undergo rearrangement process by opening the cyclopropyl to form a more stable pyran ring (labeled as F4B).

As for 8-OHNVP, it may lose the cyclopropyl radical first to generate fragment ion with m/z 242.0 (labeled as F5) as 2- and 3-OHNVP (Figure 3D). However, the 8-hydroxyl group enhances the electron density of the pyridine ring, consequently stabilizing the putative carbonyl cation to attenuate its tendency to lose carbon monoxide moiety as demonstrated by the low abundance of the peak at m/z 214.1. When the carbonyl oxygen is protonated, it can also undergo similar fragmentation pathway as for 2- and 3-OHNVP as shown in Figure

3E. The 8-OH group remained in the cation, resulting in the product ion (labeled as F6A and F6B) at m/z 177.0 instead of 161.0.

The high accurate mass spectra of all the four hydroxyl metabolites were also obtained on the LTQ Orbitrap XL (Thermo Fisher Scientific Corporation, San Jose, CA) mass spectrometer in Campus Chemical Instrumental Center (CCIC). The observed exact mass and calculated mass of all the fragments were listed in Table 1. The high mass accuracies of the protonated molecular ions and fragmentation ions (labeled in Figure 3) (less than 5 ppm) indicated that the elementary compositions of these ions are consistent with the proposed structures. Therefore, a postulated fragmentation pathway for NVP and its five metabolites was inset on the left side of their respectively CID spectra as shown in Figure 1.

As shown, 2-OHNVP and 3-OHNVP will interfere with each other and they all interfere in the detection of 8-OHNVP, if they are not separated by chromatography. In order to remove these interferences from each other, we used a gradient elution as described in experimental section instead of isocratic elution as reported in our previous method. (Liu et al., 2007) Under this condition, all these three isomers were separated from each other with 2-OHNVP eluting at 13.36 min, 3-OHNVP eluting at 17.08 min, and 8-OHNVP eluting at 15.54 min (Figure 4H). The other two metabolites 12-OHNVP eluted at 14.36 min and 4-CANVP eluted at 22.65 min (Figure 4H, 100 ng/mL each). The difference in elution time of these isomers may be attributed to their difference tendency to form intramolecular hydrogen bonds result in their different proton affinities. Nevirapine eluted at 23.53 min and the internal standard hesperetin eluted at 25.31 min. The extracted ion chromatograms (XIC) of NVP and its five metabolites at LLOQ level were shown in Figures 4A–4F. The shaded area was the integration of the peak of interest, which all showed good signal to noise ratio. Although the peaks corresponding to 2-, and 3-OHNVP were observed in the extracted ion chromatogram of 8-OHNVP, they were separated by retention time and quantification of 8-OHNVP could be obtained with high accuracy (Figure 4C). No peaks were observed in the same retention time range in baboon serum blank, which indicated the high specificity of this assay.

Assay Validation in Baboon Serum

Our recently developed LC-MS/MS method for simultaneous quantification of NVP, 2-OHNVP and 4-CANVP demonstrated that ethyl acetate extraction of NVP, 2-OHNVP and 4-CANVP from baboon serum is very efficient. In addition to these three species, it is expected three other hydroxylated NVP isomers 3-OHNVP, 8-OHNVP and 12-OHNVP could also be extracted from baboon serum. Therefore, we adapted the same ethyl acetate extraction method to extract NVP and its five oxidized metabolites from baboon serum. The recovery yields of 3-OHNVP, 8-OHNVP and 12-OHNVP were found to be similar to those of NVP, 2-OHNVP and 4-CANVP shown in our previous method. (Liu et al., 2007) Ten calibration standards (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL) were prepared using the protocols described in the experimental section with the concentration ranging from 1.0 ng/mL to 1000 ng/mL. Six replicates were prepared for the quality control (QC) data points (1, 5, 50 and 500 ng/mL) and the within-run precision and accuracy for NVP and its five metabolites are listed in Table 1. No signal was detected for 4-CANVP at 1 ng/mL and the LLOQ of 4-CANVP was determined to be 5 ng/mL. While using the MRM channel of 297.07>278.83 to monitor 4-CANVP, there existed an interference peak in baboon serum, especially at low concentrations. Therefore, the MRM channel 297.07>209.84 was selected to quantify 4-CANVP since there was essentially no interference peak under this condition. For 4-CANVP, the calibration curve was linear from 5 ng/mL to 1000 ng/mL with a regression coefficient (r^2) of 0.994 by use of 0.10 mL baboon serum. The within-run coefficients of variance at QC data points 5, 50 and 500 ng/mL were 8.03%, 9.99% and 7.48%, respectively. The accuracies at the above QC data points were 109.35%, 96.52% and

96.01%, respectively. The calibration curves for NVP and other four metabolites (2-, 3-, 8-, and 12-OHNVP) were linear from 1 to 1000 ng/mL with the regression coefficients > 0.998. The within-run coefficients of variance at QC data points (1 ng/mL, 5 ng/mL, 50 ng/mL and 500 ng/mL) were below 13% and their accuracies were in the range of 91% to 107% (Table 1). The between run (n=6) precision for NVP and its five metabolites ranged from 2% to 14% and the accuracies ranged from 93% to 114% accuracies except that of LLOQ for 2-OHNVP, which had an accuracy of 115.81% for between-run validation.

Pharmacokinetics of NVP and Its Five Metabolites in Baboons

With available serum samples from a single-dose escalation study to determine the required dose to achieve the comparable serum concentrations of NVP in the similar range of human serum (plasma) in eight non-pregnant baboons, the PK profiles of NVP and its metabolites at predose, 1.5, 4 and 8 hrs post dose were evaluated using this method. No 8-OHNVP was detected in all of these samples. One possible reason is that most of the metabolite may exist in the form of glucuronide as demonstrated in human plasma.(Riska et al., 1999b) The mean serum concentration-time profiles of NVP and its four other metabolites (2-OHNVP, 3-OHNVP, 4-CANVP and 12-OHNVP) are shown in Figures 5A to E. The mean C_{max} and AUC_{0-8h} of NVP and its metabolites at doses 5 and 10 mg/kg of NVP in a solution formulation and 400 and 800 mg total of NVP in a tablet formulation are listed in Table 3. The parent NVP compound was the major species detected in baboon serum following single dose administration. The major metabolites detected were 4-CANVP and 12-OHNVP. Lower levels of 3-OHNVP and 2-OHNVP were observed as compared to 12-OHNVP and 4-CANVP for all dosing groups. Previously, 2-OHNVP and 3-OHNVP were quantified at a composite and now quantified individually with this assay. As a result, it was found that 3-OHNVP was more abundant than 2-OHNVP. The metabolic profile of NVP in baboon is consistent with a previous study of the biotransformation of NVP in monkey⁵. Also it was noticed that that solution formulation of NVP yield higher plasma level (594 ng/mL for 10 mg/kg, corresponding to about 200 mg) than that of the tabulate formulation (328.3 ng/mL for 400 mg NVP). Previous study demonstrated that there is no significant difference in the absorption and bioavailability of NVP in solution and tablets. Therefore, it seems that foods like banana or meshed bread may have some effects on the pharmacokinetics of NVP in baboon. It is worth mentioning that the ratio of the plasma level of 12-OHNVP and 4-CANVP at the four doses is 0.21, 1.3, 0.6, and 1.2, respectively and the larger the ratio is, the higher the plasma level of 12-OHNVP is, the ratio of 12-OHNVP and 4-CANVP is quite stable when it is ≥ 1 . This result suggest that conversion of 12-OHNVP to 4-CANVP is dependent on the plasma level of 12-OHNVP and then reach a steady status when the plasma level of 12-OHNVP reaches a threshold concentration of these enzymes. The metabolic profile of NVP in baboon was found to be different from that in human plasma, in which the major metabolites detected were 3-OHNVP followed by 12-OHNVP and 2-OHNVP and a low plasma level of 8-OHNVP, which was also detected in human plasma.(Rowland et al., 2007) Further investigation is warranted to find out the molecular basis of the different metabolic profile of NVP in human plasma and baboon serum.

Conclusion

An ultra sensitive and specific LC-MS/MS method for simultaneous quantification of NVP and its five metabolites in baboon serum was established and validated. Using the method, not only was the pharmacokinetics of NVP and 4-CANVP characterized, but also the pharmacokinetics of all four hydroxylated NVPs was profiled. Although recently an LC-MS/MS assay to quantify the five metabolites of NVP simultaneously in human plasma was reported,(Rowland et al., 2007) our method differs from a recently published LC-MS/MS

assay to simultaneously quantify five NVP metabolites in the following aspects: (1) our method can quantify not only the five NVP metabolites, but also NVP itself; (2) our method showed improved sensitivity 10 fold for the four hydroxyl metabolites and 2-fold for the 4-CANVP; (3) our matrices is baboon serum, not human plasma; (4) the monitor SRM channel for 4-CANVP is 297.1>209.8, not 297.1>278.8 to avoid potential matrices interference. In addition, the detailed fragmentation pathways of the four hydroxyl nevirapine metabolite isomers were proposed and discussed. The successful application of this assay to pharmacokinetic study of NVP in baboons indicated that this assay was reliable and robust and it is the first for us to characterize the time profile of five NVP metabolites in baboon. Further studies by this method are still on-going to evaluate the association between the serum levels of NVP and its metabolites during pregnancy in olive baboons and potentially in human plasma to understand the potential correlation of the metabolism of NVP, polymorphism of CYP 2B6, and hepatotoxicity.

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Figure 1.

Tandem mass spectra of NVP and its five metabolites. **A.** the collision induced dissociation (CID) spectrum of the protonated molecular ion MH^+ of m/z 267.01 of NVP exhibits a base peak at m/z 226.01; **B.** the CID of MH^+ of m/z 283.00 of 2-OHNVP exhibits a base peak at m/z 161.11; **C.** the CID of MH^+ of m/z 283.01 of 3-OHNVP exhibits a base peak at m/z 214.00; **D.** the CID of MH^+ of m/z 297.07 of 4-CANVP exhibits two fragment peaks at m/z 209.07 and 278.83; **E.** the CID of MH^+ of m/z 283.05 of 8-OH exhibits a base peak at m/z 241.90; **F.** the CID of MH^+ of m/z 283.03 of 12-OH exhibits a base peak at m/z 265.00. A postulated fragment pathway for each compound is inset in their respective CID spectrum.

Figure 2. Collision energy dependent parent ion and fragmental ion intensities of 2-OHNVP (**A**), 3-OHNVP (**B**) and 8-OHNVP (**C**) as indicated in the relative percentage of the ions to the total ion intensity.

Figure 3.

The putative fragmentation pathway for 12-OHNVP (**A**); 2- and 3-OHNVP to generate product ions at m/z 214.1 (**B**), 161.1 (**C**) and 8-OHNVP to generate the product ion at m/z 242.1 (**D**) and 177.0 (**E**). The hydroxyl group in the middle indicates that the hydroxyl group can be either located in carbon 2 or 3 positions.

Figure 4.

The extracted ion chromatograms (XICs) of 2-OHNV with the reaction channel 283.00>161.11 at collision energy 26% (**A**), 3-OHNV with the reaction channel 283.11>214.00 at collision energy 30% (**B**), 8-OHNV with the reaction channel 283.12>241.90 at collision energy 27% (**C**), 4-CANV with the reaction channel 297.07>209.84 at collision energy 30% (**D**), 12-OHNV with the reaction channel 283.03>265.00 at collision energy 20% (**E**) and NVP with the reaction channel 267.01>226.01 at collision energy 30% (**F**). The shaded area indicated the integration from Xcalibur software, which was used for quantification. NVP and its four hydroxyl metabolites were monitored at LLOQ of 1.0 ng/mL and 4-CANV was monitored at LLOQ of 5.0 ng/mL. The total ionic chromatogram (TIC) of the blank baboon serum was shown in (**G**) and the TIC of the mixtures of internal standard, nevirapine and its five metabolites under gradient elution was shown in (**H**) (100 ng/mL each). The shaded area indicated the integration from Xcalibur software, which was used for quantification. (AA: integration area, RT: retention time, SN: signal to noise ratio)

Figure 5. The mean plasma concentration-time profiles of NVP (A), 2-OHNVP (B), 3-OHNVP (C), 4-CANVP (D), and 12-OHNVP (E) in baboon serum following oral doses at 5 mg/kg, 10 mg/kg, 400 mg/kg and 800 mg/kg NVP.

Table 1

High resolution mass spectra of NVP and its four hydroxyl metabolites by LTQ Orbitrap XL mass spectrometer. M: molecular ion; F: fragmentation ion. The structures of the ions are depicted in Figure 3.

	Formula	Fragment	Calculated Mass	Observed Mass	% Error (ppm)
12-OHNVVP	C ₁₅ H ₁₅ N ₄ O ₂ ⁺	M1	283.1190	283.1181	3.18
	C ₁₅ H ₁₃ N ₄ O ⁺	F1A/F1B	265.1084	265.1075	3.39
2-OHNVVP/3-OHNVVP	C ₁₅ H ₁₅ N ₄ O ₂ ⁺	M2	283.1190	283.1180	3.53
	C ₁₂ H ₁₀ N ₄ O ₂ ⁺	F2	242.0798	242.0792	2.48
	C ₁₁ H ₁₀ N ₄ O ⁺	F4	214.0849	214.0843	2.80
	C ₉ H ₉ N ₂ O ⁺	F6A/F6B	161.0709	161.0702	4.35
8-OHNVVP	C ₁₅ H ₁₅ N ₄ O ₂ ⁺	M3	283.1190	283.1179	3.89
	C ₁₂ H ₁₀ N ₄ O ₂ ⁺	F3	242.0798	242.0792	2.48
	C ₁₁ H ₁₀ N ₄ O ⁺	F5	214.0849	214.0843	2.80
	C ₉ H ₉ N ₂ O ₂ ⁺	F7A/F7B	177.0659	177.0651	4.52

Table 2

Within-run validation data of NVP and its metabolites in baboon serum by LC-MS/MS (n=6).

Conc. (ng/mL)		NVP	2-OHNVP	3-OHNVP	4-CANVP	8-OHNVP	12-OHNVP
1	Ave.	1.02	0.93	0.98		0.91	1.03
	STD	0.08	0.07	0.06		0.07	0.07
	C.V./%	7.61	7.86	6.42		8.00	6.38
	Acc. ^a %	101.85	92.94	97.84		91.45	103.23
5	Ave.	4.72	4.72	4.68	5.47	4.91	4.71
	STD	0.59	0.47	0.27	0.44	0.53	0.50
	C.V./%	12.56	9.97	5.78	8.03	10.74	10.60
	Acc. %	94.45	94.44	93.64	109.35	98.13	94.10
50	Ave.	51.14	52.04	49.84	48.26	47.94	51.11
	STD	2.39	2.65	2.21	4.82	2.83	2.03
	C.V./%	4.67	5.10	4.43	9.99	5.91	3.96
	Acc. %	102.29	104.08	99.68	96.52	95.88	102.22
500	Ave.	510.84	532.87	532.61	480.04	513.66	521.20
	STD	12.21	12.35	9.52	35.92	16.24	11.62
	C.V./%	2.39	2.32	1.79	7.48	3.16	2.23
	Acc. %	102.17	106.57	106.52	96.01	102.73	104.24

Note:

^a accuracy

Table 3
Between-run validation data of NVP and its metabolites in baboon serum by LC-MS/MS (n=6).

Conc. (ng/mL)		NVP	2-OHNVP	3-OHNVP	4-CANVP	8-OHNVP	12-OHNVP
1	Ave	1.03	1.16	1.10		0.99	1.14
	STD	0.14	0.14	0.10		0.11	0.15
	C.V./%	13.92	12.38	9.29		11.46	12.70
	Acc. ^a /%	103.23	115.81	109.74		99.49	114.35
5	Ave.	5.28	5.02	4.82	5.70	4.92	5.02
	STD	0.38	0.25	0.17	0.64	0.45	0.19
	C.V./%	7.17	4.98	3.48	11.20	9.14	3.79
	Acc./%	105.52	100.35	96.38	113.92	98.31	100.50
50	Ave.	50.74	50.56	50.30	53.97	49.04	50.81
	STD	1.06	1.92	1.75	5.94	1.95	2.24
	C.V./%	2.08	3.81	3.49	11.01	3.98	4.41
	Acc./%	101.47	101.11	100.61	107.94	98.08	101.63
500	Ave.	509.13	505.80	509.55	559.60	501.40	507.48
	STD	49.60	29.64	22.68	78.85	21.85	28.65
	C.V./%	9.74	5.86	4.45	14.09	4.36	5.65
	Acc./%	101.83	101.16	101.91	111.92	100.28	101.50

Note:

^a accuracy

Table 4

Relevant pharmacokinetic parameters in non-pregnant olive baboons.

Dose	NVP		2-OHNVP		3-OHNVP		4-CANVP		12-OHNVP	
	Cmax ^a ng/mL	AUC _{0-8h} ^b ng.r/mL	Cmax ^a ng/mL	AUC _{0-8h} ^b ng.h/mL	Cmax ^a ng/mL	AUC _{0-8h} ^b ng.h/mL	Cmax ^a ng/mL	AUC _{0-8h} ^b ng.h/mL	Cmax ^a ng/mL	AUC _{0-8h} ^b ng.h/mL
5 mg/kg (n = 2)	192.12±61.51	768.55±234.59	N/A	N/A	11.50±3.63	42.38±5.66	144.90±22.31	525.64±6.59	31.38±10.59	125.20±23.54
10 mg/kg (n = 2)	593.97±371.38	2705.18±1552.29	2.82±1.03	13.20±9.62	19.69±9.94	114.11±68.95	214.34±50.64	1078.11±441.22	275.25±126.07	1308.65±891.58
400 mg (n = 2)	328.32±13.39	1942.89±57.23	2.27±0.23	13.79±0.16	16.53±1.10	100.15±9.85	263.68±16.96	1502.81±66.95	157.16±7.06	912.80±164.04
800 mg (n = 2)	908.73±657.41	5108.02±3626.20	5.60±4.34	35.41±25.40	72.85±60.37	469.36±396.51	320.92±262.82	1487.22±1110.86	385.38±346.21	2294.84±2065.77

Note:

^a maximum concentration

^b area under curve.