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ScienceDirect
Journal of Chromatography B, 856 (2007) 252–260
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitation of five nevirapine oxidative metabolites in human plasma using liquid chromatography–tandem mass spectrometry

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Received 1 March 2007; accepted 12 June 2007
Available online 22 June 2007

Abstract

A multiple-reaction-monitoring LC/MS/MS method for the analysis of nevirapine oxidative metabolites, 2-hydroxynevirapine, 3-hydroxynevirapine, 8-hydroxynevirapine, 12-hydroxynevirapine, and 4-carboxynevirapine, in human plasma was developed and validated. The metabolites were isolated from 50 μL heparinized plasma by enzymatic hydrolysis of the glucuronide conjugates to the free metabolite followed by protein precipitation with acetonitrile. Peaks were quantitated at 3.03 min for the 4-carboxynevirapine metabolite, at 3.72, 4.27, 5.27, and 5.73 min for the positional 2-hydroxynevirapine, 12-hydroxynevirapine, 3-hydroxynevirapine, and 8-hydroxynevirapine metabolites, respectively, and 2.30 min for the internal standard, pirenzepine. The assay was accurate and precise based on assay validation controls over the nominal range of 0.010–1.0 mg/L. The average accuracy at the lowest concentration quality control (QC) sample was 16% (difference from theoretical value) for 8-hydroxynevirapine, all others were closer to their known respective standards. Within- and between-day precisions were within 12% for quality control samples for all five metabolites. Repetitive thawing and freezing did not have an effect on any metabolite through a minimum of three cycles. Thawed samples, remaining in plasma for 4 h before extraction, were within 5% of theoretical value. Stability of the extracted samples on the autosampler at room temperature was evaluated for 48 h and was observed to be within 12% of a fresh analytical sample for 2-hydroxynevirapine and 3-hydroxynevirapine; other metabolites were within 6% of theoretical value. The utility of the analytical method was demonstrated using trough steady-state plasma samples collected from 48 patients in a hepatic impairment study.

Keywords: Nevirapine metabolites; 2-Hydroxynevirapine; 3-Hydroxynevirapine; 8-Hydroxynevirapine; 12-Hydroxynevirapine; 4-Carboxynevirapine

1. Introduction

Nevirapine, a non-nucleoside reverse transcriptase inhibitor [1,2] for the treatment of human immunodeficiency viral infection (HIV-1), is readily absorbed (>90%) after oral administration [3]. Nevirapine is approximately 60% bound to plasma proteins and freely partitions to all tissues including the brain. Cytochrome P450 oxidation, glucuronide (ether) conjugation, and urinary excretion of the ether-glucuronidated-oxidized-metabolites represents the primary route of nevirapine biotransformation and elimination in humans; renal excretion of unchanged drug plays a minor role [4]. Nevirapine is an inducer of hepatic cytochrome P450 (CYP) metabolic enzymes 3A4 and 2B6 [5]. Nevirapine induces CYP3A4 and CYP2B6 by approximately 20–25% each, as indicated by erythromycin breath test results and urine metabolites. Autoinduction of CYP3A4- and CYP2B6-mediated metabolism leads to an approximately 1.5–2-fold increase in the apparent oral clearance of nevirapine as treatment continues from a single dose to 2–4 weeks of dosing with 200–400 mg/day. Autoinduction also results in a corresponding decrease in the terminal phase half-life of nevirapine in plasma, from approximately 45 h (single dose) to approximately 25–30 h following multiple dosing with 200–400 mg/day.

Nevirapine is primarily cleared through oxidative drug metabolism (Phase I reaction) in the smooth endoplasmic reticulum of the hepatocytes followed by glucuronidation (Phase II reaction) in the cytosol to water-soluble conjugates for rapid

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doi:10.1016/j.jchromb.2007.06.007

elimination from the body. Therefore, clinical situations in HIV-1 therapy that influence steady-state metabolic clearance of nevirapine would result in a change in systemic exposure, as well as a shift in the metabolite profile generated. Therapeutic examples would be hepatic impairment, hepatitis co-infection, or maturation of the liver from pre-term birth to adolescence.

Nevirapine and the known oxidative metabolites [4,6] are shown in Fig. 1. To evaluate a potential shift in clearance, a liquid chromatography–tandem mass spectrometry (LC/MS/MS) assay was developed for the metabolites. An LC/MS/MS assay was necessary because four of the five metabolites are regiospecific isomers having the same molecular weight.

Because nevirapine is used in combination therapy with additional antiretrovirals and anti-infective concomitant medications, therapeutic drug monitoring with these drug combinations has proven useful to evaluate efficacy, safety, and pharmacokinetics [7–9]. Therapeutic drug monitoring and novel extensive drug combinations in acquired immune deficiency syndrome therapy over the past 10 years has produced numerous serum and plasma nevirapine assays using liquid chromatography–ultraviolet detection [10–25], gas chromatography [26], electrokinetic chromatography [27], thin-layer chromatography [28,29], liquid chromatography with tandem mass spectrometry assays [30,31], and an enzyme immunoassay [32].

This paper describes for the first time a sensitive, specific, and rapid LC/MS/MS method for the simultaneous determination of the five nevirapine metabolites in human plasma. This assay method was successfully applied to several nevirapine steady-state pharmacokinetic studies evaluating hepatic impairment, pediatric metabolic maturation from newborn to adoles-

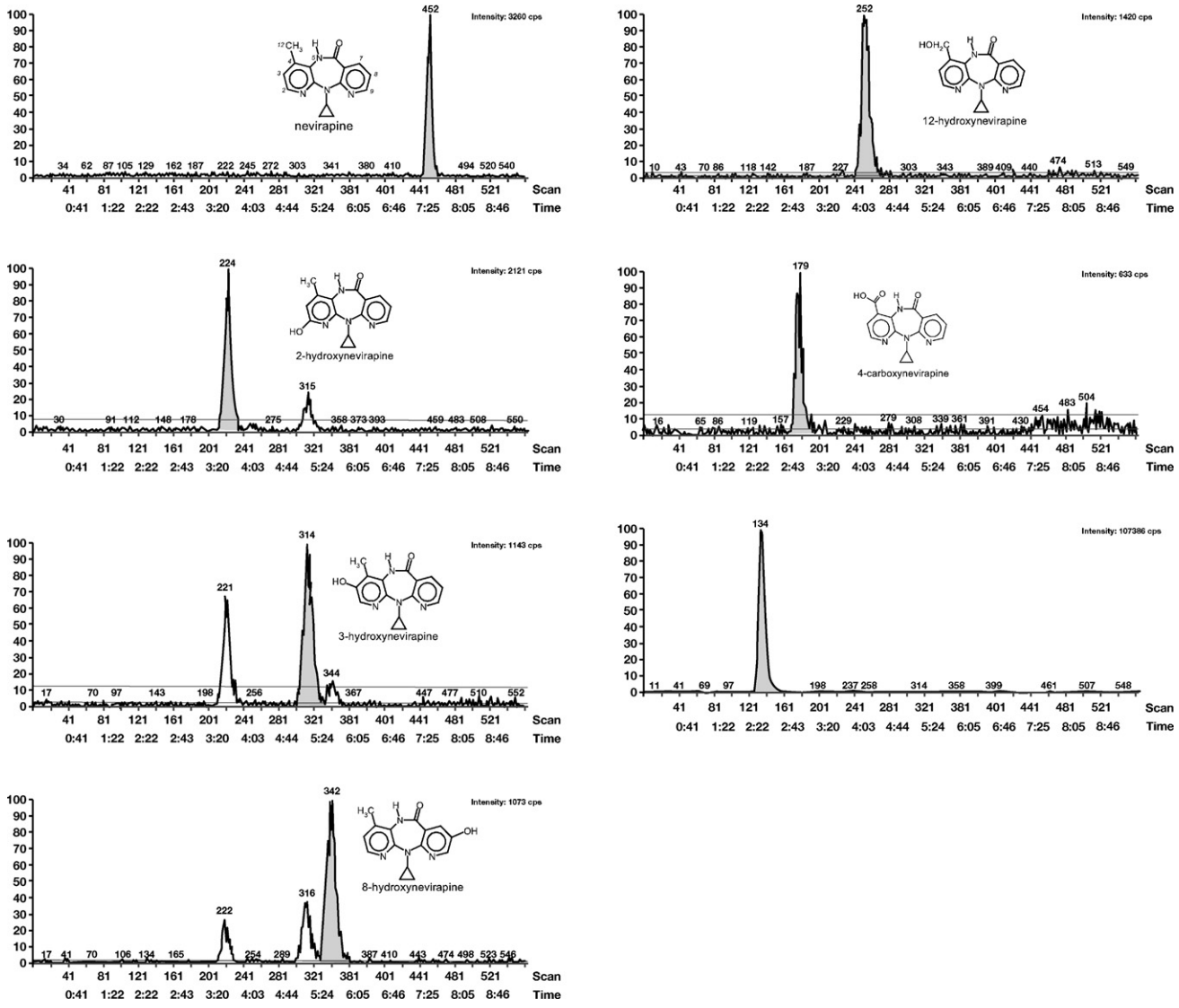

Fig. 1. Chromatograms and structures for nevirapine and the five metabolites at the limit of quantitation (10 ng/mL) for this assay in plasma. The internal standard, pirenzepine, elutes at 2.30 min (scan 134). Quantitation was performed using multiple-reaction monitoring of the reaction transitions of m/z = Q1 283.2 → Q3 160.8 for 2-hydroxynevirapine, m/z = Q1 283.2 → Q3 213.6 for 3-hydroxynevirapine, m/z = Q1 283.2 → Q3 242.0 for 8-hydroxynevirapine, m/z = Q1 283.2 → Q3 223.0 for 12-hydroxynevirapine, m/z = Q1 297.2 → Q3 278.9 for 4-carboxynevirapine, and m/z = Q1 352.3 → Q3 251.6 for the internal standard, pirenzepine.

cence, and the metabolic evaluation of spontaneous adverse events.

2. Material and methods

2.1. Standard substances and reagents

The nevirapine metabolite standards were synthesized in gram quantities as described previously [6]. Structural confirmations and purity for each of the standards are described in Table 1. HPLC-grade methanol and acetonitrile were purchased from Honeywell Burdick and Jackson (Muskegon, MI), formic acid and ammonium acetate were purchased from Mallinckrodt Baker (Phillipsburg, NJ), and water was filtered using a Continental Modulab Reverse-Osmosis System. Human plasma with sodium heparin anticoagulant was purchased as a pooled specimen from BioChemed Services (Charleston, SC) and stored at −20 °C until use. The internal standard, pirenzepine, was purchased as a ≥98% pure powder (P-7412) and the enzyme β-glucuronidase (G-8396) was purchased as a lyophilized powder, 1000 units/vial, from Sigma–Aldrich (St. Louis, MO).

2.2. Liquid chromatography operating conditions

Plasma nevirapine concentrations (1–10 mg/L) were determined by a previously validated HPLC-UV method [11]. Separation by HPLC for the five metabolites was conducted using Hewlett-Packard 1100 Series Binary Pumps (Agilent Technologies, Wilmington, DE) with a Aquasil® C_{18} (5 μm) column (2 mm i.d. × 100 mm, P/N 105-775-2, Keystone Scientific Inc., Bellefonte, PA). A mixture of aqueous 2 mM ammonium acetate (pH 3.3–3.4) with acetonitrile (80:20, v/v) was used as the mobile phase at a flow rate of 0.30 mL/min. The column was cleaned at 6.0 min with a 40:60 (v/v) mixture of the mobile phase 1.5 min followed by a 2.25 min re-equilibration with the original mobile phase. Injections (25 μL) were made with a CTC Analytics LC-PAL autosampler (Zwingen, Switzerland) using a 50 μL loop at room temperature. Under these conditions, chromatographic peaks occurred at 3.03 min for the 4-carboxynevirapine metabolite, at 3.72, 4.27, 5.27, and 5.73 min for the positional 2-hydroxynevirapine, 12-hydroxynevirapine, 3-hydroxynevirapine, and 8-hydroxynevirapine metabolites, respectively, and 2.30 min for the internal standard (Fig. 1). The parent drug nevirapine has a retention time of 7.60 min, but quantitation of unchanged drug was not the purpose of this assay.

2.3. Mass spectrometry operating conditions

A Sciex API 3000 mass spectrometer (Applied Biosystems/MDS, Foster City, CA) and the HPLC system were interfaced by using a Turbo IonSpray positive ion source with multiple-reaction-monitoring (MRM) detection. The Turbo temperature was 425 °C with the spray voltage set at 4.0 kV. Nitrogen Ultra High Purity and Zero Grade obtained from MG Industries (Malvern, PA) were used as the curtain gas and auxiliary flow gas for the Turbo (8.0 L/min), respectively. Quantitation was performed using MRM of the reaction transitions of *m/z* = Q1 283.2 → Q3 160.8 for 2-hydroxynevirapine,

Table 1
Synthetic standards used to develop nevirapine metabolite assay

Compound	NMR	MS
Nevirapine (purity: 99.9%)	Proton: NH, H9, H2, H7, H8, H3, cyclopropyl, and CH_3 at position 4 are consistent with marketed reference standard.	Solid probe EI: produced *m/z* 266 = $M^{+\bullet}$ = MW and *m/z* 265 = $(M-H)^+$ fragment consistent with structure.
2-Hydroxynevirapine (purity: 97.7%)	Proton: OH, NH, H9, H7, H8, H3, cyclopropyl, and CH_3 at position 4 are consistent with the structure. NOESY: Supports position of the hydroxy at position 2.	Solid probe EI: produced *m/z* 282 = $M^{+\bullet}$ = MW and *m/z* 281 = $(M-H)^+$ fragment consistent with structure.
3-Hydroxynevirapine (purity: 93.6%)	Proton: NH, OH, H9, H7, H2, H8, cyclopropyl, and CH_3 at position 4 are consistent with the structure. NOESY: Supports position of the hydroxy at position 3.	Solid probe EI: produced *m/z* 282 = $M^{+\bullet}$ = MW and *m/z* 281 = $(M-H)^+$ fragment consistent with structure. Minor phthalate impurity.
8-Hydroxynevirapine (purity: 94.2%)	Proton: OH, NH, H2, H9, H7, H3, cyclopropyl, and CH_3 at position 4 are consistent with the structure. NOESY: Supports position of the hydroxy at position 8.	Solid probe EI: produced *m/z* 282 = M+• = MW and *m/z* 281 = (M − H)+ fragment consistent with structure.
12-Hydroxynevirapine (purity: 99.5%)	Proton: NH, H9, H2, H7, H3, H8, CH_2, and cyclopropyl are consistent with the structure. NOESY: Supports position of the hydroxy at position 12.	Solid probe EI: produced *m/z* 282 = M+• = MW and *m/z* 281 = (M − H)+ fragment consistent with structure.
4-Carboxynevirapine (purity: 94.6%)	Proton: NH, H9, H2, H7, H3, H8, and cyclopropyl are consistent with the structure. Coupling constants of COOH at 15–13 ppm consistent with structure. HMBC experiment verified carboxy at position 4.	Solid probe EI: produced *m/z* 296 = M+• = MW and *m/z* 295 = (M − H)+ fragment consistent with structure. Minor impurity at *m/z* 256 (sulfur)

NOESY = Nuclear Overhauser Enhancement Spectroscopy.
HMBC = Heteronuclear Multiple Bond Connectivity.

m/z = Q1 283.2 → Q3 213.6 for 3-hydroxynevirapine, m/z = Q1 283.2 → Q3 242.0 for 8-hydroxynevirapine, m/z = Q1 283.2 → Q3 223.0 for 12-hydroxynevirapine, m/z = Q1 297.2 → Q3 278.9 for 4-carboxynevirapine, and m/z = Q1 352.3 → Q3 251.6 for the internal standard, pirenzepine. The reaction transition of m/z = Q1 267.2 → Q3 225.9 for nevirapine was not used. Dwell times were 150 ms for each reaction transition except the internal standard, which only used 75 ms. Tuning was conducted with a 250 ng/mL standard solution of the metabolites infused in methanol and mobile phase at a rate of 0.20 mL/min.

2.4. Data acquisition

The data system was configured to automatically calculate and annotate the areas of the metabolites of nevirapine and the internal standard (pirenzepine). Calibration curves were constructed using peak area ratios (PARs) of the calibration samples by applying a quadratic-weighted 1/concentration squared regression analysis. All concentrations were then calculated from their PARs against the calibration line.

2.5. Preparation of stock and standard solutions

A 250 mL stock solution of 1 M ammonium acetate was prepared every 6 months with filtered water and stored at 0–4 °C. The mobile phase concentration of 2 mM ammonium acetate was prepared weekly by dilution and adjusting the pH to 3.3–3.4 with formic acid. The mobile phase was prepared by combining 800 mL of the 2 mM ammonium acetate solution with 200 mL of acetonitrile. The β-glucuronidase solution was prepared by addition of 10.0 mL filtered water to a vial of 1000 units lyophilized powder and vortexing until dissolved. This enzyme solution was stable for up to 3 days at 0–4 °C. Two nevirapine metabolite stock solutions were prepared in methanol at a nominal concentration of 0.100 mg/mL from separate weighings of the reference standards using unsilylated glassware. The stock solutions were store at 0–4 °C when not in use and expired 2 weeks after date of preparation. The internal standard stock solution of pirenzepine was prepared in filtered water at a nominal concentration of 1.00 mg/mL using unsilylated glassware. The internal standard stock solution was store at 0–4 °C when not in use and expired 36 days after date of preparation [33]. This internal standard stock solution was diluted to 10 μg/mL for use in the assay and was stored in the same manner as the stock solution.

2.6. Standard samples for calibration curve and for QC samples

Calibration standards were prepared from one of the nevirapine metabolites stock solutions prepared (or aliquots of previously prepared calibration pools) to span the calibration range. Suggested calibration levels were 10.0, 25.0, 50.0, 100, 250, 500, and 1000 ng/mL. After preparation, aliquots of the calibration standards were stored in polypropylene tubes at ≤−20 °C until use. Quality control (QC) samples are prepared from a high QC pool in plasma (1000 ng/mL) that was prepared using the second nevirapine metabolites stock solution. Suggested QC levels were 10.0, 25.0, 150, and 750 ng/mL. After preparation, aliquots of quality control pools were stored in polypropylene tubes at ≤−20 °C until use.

2.7. Procedure for plasma sample preparation

A reagent blank (no plasma or internal standard), plasma blank (no internal standard), and a plasma blank with internal standard were prepared and extracted with each analytical run.

Frozen plasma samples were thawed in a cold-water bath, vortexed, and 50 μL was transferred to a polypropylene microcentrifuge tube. β-Glucuronidase (50 μL of the 100 units/mL stock solution) was added to each tube, capped, mixed well, and incubated overnight in a water bath at 37 °C. In the morning, the samples were cooled to room temperature, vortex mixed, and 25 μL of the 10 μg/mL internal standard spiking solution was added to all samples. Acetonitrile (0.250 mL) was added to each sample, capped, mixed for 1 min, and then centrifuged at 14,000 rpm for 5 min. A 50 μL aliquot of the supernatant was added to a polypropylene autosampler vial containing 250 μL mobile phase, capped, and vortex mixed briefly. The calibration standards and quality control samples were prepared in an identical manner with the exception of the overnight incubation step.

2.8. Method validation

The linearity of this procedure was evaluated by analyzing seven calibration standards in duplicate on each validation run. The calibration standard concentrations spanned the range of 10.0–1000 ng/mL for the metabolites of nevirapine. A quadratic, 1/concentration squared ($1/C^2$) weighted least-square regression algorithm was used to plot the chromatographic peak area ratio of the metabolites (2-hydroxynevirapine, 3-hydroxynevirapine, 8-hydroxynevirapine, 12-hydroxynevirapine, and 4-carboxynevirapine) to internal standard versus theoretical concentration. Linearity, average back-calculated values, and reproducibility were then calculated. The lower limit of quantitation (LOQ) was the lowest non-zero concentration level (Fig. 1) which could be quantitated accurately and reproducibly. Precision and accuracy were determined by the analysis of quality control pools, one prepared at the lower limit of quantitation and three additional pools prepared at concentrations spanning the calibration range. Precision was expressed as the percent coefficient of variation (%CV) of each pool. Accuracy was measured as the percent difference from theoretical. Intra-assay precision and accuracy were evaluated by multiple analyses (n = 6) of the quality control pools during one of the validation runs. Inter-assay precision and accuracy were evaluated by multiple analyses of the quality control pools during each validation run. Relative recovery was evaluated by comparing responses from precipitated quality controls to external solutions of the analytes prepared in filtered water. Freeze/thaw stability was evaluated by analyzing low- and high-concentration quality control samples which were thawed and refrozen three times before analysis. Thawed matrix stability in human plasma was evaluated by analyzing low- and

high-concentration quality control samples which were thawed at room temperature and allowed to remain on the laboratory bench top for approximately 4 h prior to the extraction. Re-injection process stability was evaluated by re-injecting two complete runs. The samples were stored for at least 48 h at room temperature after extraction and before re-injection. Human plasma samples from six different individuals were extracted and analyzed for the metabolites during one validation run.

Three replicates each of human plasma pools containing either 2-hydroxynevirapine, 3-hydroxynevirapine, 8-hydroxynevirapine, 12-hydroxynevirapine, or 4-carboxynevirapine at five times the highest calibration standard concentration (5 μg/mL) were extracted without adding internal standard and analyzed for analyte cross-interference.

2.9. Application to pharmacokinetic study

The assay of the nevirapine metabolites was applied to a study with hepatically impaired patients, as measured by liver fibrosis, that were already on chronic (>6 weeks exposure) nevirapine (Viramune®) 200 mg twice-daily therapy, and therefore were at steady state. Patients were stratified by Ishak score indicating mild, moderate, or severe fibrosis (with cirrhosis) to evaluate if this population might be a candidate for a dose reduction. Plasma samples were assayed for the nevirapine metabolites, following glucuronidase treatment, to evaluate if any increased nevirapine concentrations were due to the inability of CYP3A4 or CYP2B6 isozymes to metabolize the parent drug or to prevent the rate transfer of elimination of the glucuronidated product of oxidation. There were 48 evaluable pharmacokinetic patients in the trial represented by 150 plasma specimens at the analytical laboratory for purposes of estimating AUC and clearance over a 12-h period. The majority (34/48) of patients had four samples drawn to estimate exposure, clearance, and maximum plasma nevirapine metabolite levels obtained at steady state.

3. Results and discussion

3.1. Separation, specificity, sensitivity, and calibration curve range

The method is summarized briefly as follows: The metabolites of nevirapine and an internal standard, pirenzepine, were isolated from human plasma by enzyme hydrolysis followed by protein precipitation using acetonitrile. A portion of the supernatant was diluted with ammonium acetate buffer and the samples were analyzed by LC/MS/MS using positive-ion Turbo IonSpray and multiple-reaction monitoring (MRM). This method was validated with a nominal concentration range of 10.0 ng/mL to 1.0 μg/mL for the metabolites of nevirapine, using a 50 μL aliquot of human plasma. Samples collected in the clinic were stable when stored at −20 °C until analysis.

The chromatographic peaks (Fig. 1) occurred at 3.03 min for the 4-carboxynevirapine metabolite at 3.72, 4.27, 5.27, and 5.73 min for the positional 2-hydroxynevirapine, 12-hydroxynevirapine, 3-hydroxynevirapine, and 8-hydroxynevirapine metabolites, respectively, and 2.30 min for the internal standard. Under the conditions of this assay, the parent drug nevirapine elutes at 7.60 min. Because four of the five metabolites are regio-specific isomers having the same molecular weight, chromatographic separation coupled with multiple-reaction monitoring of different transitions was required to get accurate and precise quantitation of the metabolites in the presence of each other. It has previously been shown that all five metabolites are present in the plasma of patients taking nevirapine chronically [4,5].

During the test for cross-analyte interference, the contribution of the metabolite 4-carboxynevirapine at 5 μg/mL to the response of 12-hydroxynevirapine was determined to be approximately 41% of the lowest calibration level for 12-hydroxynevirapine (10.0 ng/mL). The contribution from 4-carboxynevirapine to 12-hydroxynevirapine may be due to a low-level impurity in the 4-carboxynevirapine reference standard material because 12-hydroxynevirapine is a synthetic precursor [6]. Since the levels evaluated were five times the highest calibration standard level and equal concentrations of the analytes are used in the preparation of calibration and quality control pools, this impurity does not impact the accuracy of the quantitation for 12-hydroxynevirapine. No other metabolite standard interfered with the quantitation of another metabolite at any level. Additionally, no significant endogenous chromatographic peaks were observed for the monitored masses at the retention times of 2-hydroxynevirapine, 3-hydroxynevirapine, 8-hydroxynevirapine, 12-hydroxynevirapine, and 4-carboxynevirapine, or the internal standard pirenzepine.

The assay procedure was calibrated using standard concentrations that spanned the expected therapeutic range of 10.0–1000 ng/mL for the metabolites of nevirapine. A quadratic,

Table 2
Average back-calculated calibration standards for nevirapine metabolites ($N = 6$)

Nominal concentration	2-Hydroxynevirapine		3-Hydroxynevirapine		8-Hydroxynevirapine		12-Hydroxynevirapine		4-Carboxynevirapine	
(ng/mL)	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV
10.0	9.92	6.3	9.84	2.3	9.84	1.8	9.88	4.7	10.0	5.9
25.0	25.5	3.2	26.0	2.2	25.9	3.1	25.6	4.0	25.0	2.6
50.0	50.1	4.0	50.4	3.5	51.1	4.4	50.9	3.5	49.4	2.7
100	99.9	3.7	99.2	5.2	98.5	6.9	99.3	3.3	102	4.0
250	249	3.6	248	3.3	245	4.1	247	4.2	252	3.1
500	487	3.7	474	4.3	478	5.0	481	3.5	491	4.0
1000	1020	3.1	1060	4.2	1070	7.4	1040	3.4	1010	4.0

Table 3
Intra-assay precision and accuracy for nevirapine metabolites ($N = 6$)

Nominal concentration	2-Hydroxynevirapine			3-Hydroxynevirapine			8-Hydroxynevirapine			12-Hydroxynevirapine			4-Carboxynevirapine		
(ng/mL)	Mean	%Δ from theoretical	%CV	Mean	%Δ from theoretical	%CV	Mean	%Δ from theoretical	%CV	Mean	%Δ from theoretical	%CV	Mean	%Δ from theoretical	%CV
10.0	8.68	−13.2	10.0	9.35	−6.5	4.2	8.36	−16.4	7.2	8.74	−12.6	11.0	8.98	−10.2	7.7
25.0	22.7	−9.1	3.4	24.4	−2.6	2.1	24.0	−4.2	3.3	25.3	+1.3	3.0	23.8	−5.0	4.9
150	137	−8.7	3.6	141	−5.9	5.6	142	−5.5	6.3	154	+2.9	3.5	140	−6.6	4.4
750	683	−8.9	4.7	684	−8.8	3.9	684	−8.8	7.3	750	−0.0	5.7	691	−7.8	5.6

Table 4
Inter-assay precision and accuracy for nevirapine metabolites ($N = 12$)

@	Nominalconcentration	2-Hydroxynevirapine			3-Hydroxynevirapine			8-Hydroxynevirapine			12-Hydroxynevirapine			4-Carboxynevirapine		
(ng/mL)		Mean	%Δ from theoretical	%CV	Mean	%Δ from theoretical	%CV	Mean	%Δ from theoretical	%CV	Mean	%Δ from theoretical	%CV	Mean	%Δ from theoretical	%CV
10.0		9.06	−9.5	8.1	9.58	−4.2	4.7	9.39	−6.1	9.2	9.66	−3.4	6.6	9.60	−4.0	11.9
25.0		23.3	−6.8	3.5	24.5	−1.8	3.3	24.2	−3.2	5.6	25.7	+3.0	5.0	23.5	−6.1	7.7
150		139	−7.5	5.6	143	−4.9	7.5	150	−0.2	5.7	149	−0.5	8.4	145	−3.1	5.8
750		691	−7.8	3.9	701	−6.6	4.1	727	−3.1	4.4	750	+0.0	3.4	722	−3.7	4.1

Table 5
Sample recovery and stability across the range of concentrations studied ($N = 6$)

Parameter	2-Hydroxynevirapine			3-Hydroxynevirapine			8-Hydroxynevirapine			12-Hydroxynevirapine			4-Carboxynevirapine		
	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
Relative recovery (%) plasma/water	100	–	94	110	–	102	106	–	99	105	–	96	108	–	94
Freeze/thaw 3× (% Δ from initial)	1.8	–	1.3	3.6	–	2.4	9.0	–	3.7	4.6	–	2.7	7.4	–	3.9
Thawed matrix 4 h (% Δ from initial)	−2.3	–	4.4	0.2	–	−1.0	2.4	–	0.4	−2.9	–	1.2	−2.4	–	−1.3
Process stability 48 h (% Δ from initial)	−10.6	−8.3	−4.8	−5.5	−11.5	1.6	−4.6	−2.5	−5.5	−0.3	−2.6	−1.6	−2.7	−3.9	−2.0

Low: mean value at 25.0 ng/mL from Table 3.
Mid: mean value at 150 ng/mL from Table 3.
High: mean value at 750 ng/mL from Table 3.

1/concentration squared ($1/C^2$) weighted least-square regression algorithm was used to plot the chromatographic peak area ratio of each of the metabolites to the internal standard versus theoretical concentration. The average back-calculated calibration standards and reproducibility from each level of the calibration curve are presented in Table 2. The back-calculated averages were close to the expected theoretical value, the %CV was less than 7%, and there was not a significant bias in any one analyte compared to the values for the other metabolites, indicating complete separation and independent quantitation. For this validation, the limit of quantitation was 10.0 ng/mL for each of the metabolites based on the lowest calibration and quality control standards. This was sufficient to quantitate the majority of metabolite concentrations observed *in vivo*.

3.2. Precision and accuracy

Precision and accuracy were determined by the analysis of quality control pools. The intra-assay precision and accuracy evaluated by multiple analyses ($n = 6$) of the four quality control pools (10.0, 25.0, 150, and 750 ng/mL) during one of the validation runs are presented in Table 3. The largest variability (8–11%) was observed in the lowest standard of 10.0 ng/mL. The largest deviation from theoretical (−16%) was also observed for 8-hydroxynevirapine at this lowest concentration. The inter-assay precision and accuracy evaluated by multiple analyses of the quality control pools during each validation run are presented in Table 4. Inter-assay precision was within 12% at the lowest concentration and within 10% of theoretical value, with 2-hydroxynevirapine demonstrating the largest deviation.

3.3. Extraction recovery and matrix effect

Relative recovery, evaluated by comparing responses from acetonitrile-precipitated quality control plasma specimens to solutions of the analytes prepared in filtered water, is presented in Table 5 at low and high concentrations. Relative recovery approached 100%, demonstrating that addition of acetonitrile, mixing, and centrifugation at 14,000 rpm for 5 min extracts the nevirapine metabolites completely into the supernatant. Following this clean-up method, no significant endogenous chromatographic peaks using human plasma samples from six different individuals were observed for the monitored masses at the retention times of 2-hydroxynevirapine, 3-hydroxynevirapine, 8-hydroxynevirapine, 12-hydroxynevirapine, and 4-carboxynevirapine, or the internal standard pirenzepine.

3.4. Stability

The stability assessments of the metabolites in a variety of conditions anticipated when clinical samples are received and processed are presented in Table 5 using low (25 ng/mL) and high (750 ng/mL) concentrations. All five metabolites are stable through multiple re-freezing and re-thawing cycles, while sitting

Table 6
Analysis of trough steady-state plasma samples collected from 48 patients in a hepatic impairment study demonstrating the utility of the method

Nevirapine metabolite	CYP pathway(s) involved	*n*	Geometric mean concentration (ng/mL)	Range (ng/mL)
2-Hydroxynevirapine	CYP3A4	47	186	61–453
3-Hydroxynevirapine	CYP2B6	47	646	65–2160
8-Hydroxynevirapine	CYP3A4 and CYP2D6	14	29	0–59
12-Hydroxynevirapine	CYP3A4 and CYP2D6	48	483	16–2010
4-Carboxynevirapine	Secondary oxidation	27	18	0–62

Geometric mean calculated only on specimens >0 ng/mL.

on the laboratory bench for 4 h waiting to be extracted, and while sitting at room temperature after extraction for 48 h before sample injection or re-injection.

3.5. Application of the assay method to pharmacokinetic study

A radiolabelled mass balance study in normal volunteers demonstrated that nevirapine is metabolized to five major metabolites and less than 5% of the oral dose is excreted unchanged in the urine [4]. The 2-hydroxy, 3-hydroxy, and 12-hydroxy metabolites of nevirapine are excreted in the urine at 22.9, 33.1, and 29.7%, respectively. The 8-hydroxy and 4-carboxy metabolites are excreted to a lesser extent at 3.2 and 0.7%, respectively. Therefore, measurements of these metabolites in plasma collected at the trough time of steady-state dosing from 48 patients should follow the pattern 3-hydroxynevirapine > 12-hydroxynevirapine > 2-hydroxynevirapine > 8-hydroxynevirapine ≥ 4-carboxynevirapine if metabolic patterns were unaltered by hepatic impairment or fibrosis. Nearly all patient trough samples contained the three major metabolites, 2-hydroxynevirapine, 3-hydroxynevirapine, and 12-hydroxynevirapine. The two minor metabolites, 8-hydroxynevirapine and 4-carboxynevirapine, were quantitated in only 29% (14/48) and 56% (27/48) of the patient trough samples, respectively. As can be seen in Table 6, the data observed in this trial was consistent with the ratios observed in the normal volunteer mass balance study [4]. Since these metabolites require different cytochrome P450 pathways, these pathways must be intact and functioning in the same relative manner as in normal volunteers even in the presence of hepatic fibrosis. This demonstrates the utility of an assay that can measure all five metabolites simultaneously.

4. Conclusions

A bioanalytical method for the simultaneous analysis of nevirapine oxidative metabolites, 2-hydroxynevirapine, 3-hydroxynevirapine, 8-hydroxynevirapine, 12-hydroxynevirapine, and 4-carboxynevirapine, in human plasma was developed and validated. The method is robust for the determination of each metabolite in the presence of each other as observed in clinical therapy. The analytes can be reliably quantitated to a lower limit of 10 ng/mL and are stable under a variety of conditions anticipated when clinical samples are received and processed in a typical LC/MS/MS laboratory. The method has been successfully used in a clinical trial to monitor the metabolic patterns observed when dosing nevirapine in the presence of hepatic impairment or fibrosis.

Acknowledgements

The authors wish to thank Anna Maria Cammett, M.Sc., Peter Piliero, M.D., and Stephen Storfer, M.D., for their assistance in the clinical portion of this study.

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