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Subnanogram on-line column-switching liquid chromatographic–tandem mass spectrometric quantification method for nelfinavir and methoxyphenol metabolite M1 in rat plasma

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Abstract

A new on-line, rapid and sensitive column-switch LC/MS/MS method to measure nelfinavir (NFV), an HIV-1 protease inhibitor, and its major metabolite (M1) in rat plasma was developed. Rat plasma containing the analytes and the internal standard was treated with acetonitrile and the supernatant was processed through an on-line extraction and an analytical columns, with a column-switch device. ESI-LC/MS with multiple reaction monitors for appropriate analytes was performed. This assay gave a limit of quantitation (LOQ) of <1 ng/mL for the analytes with 5 min run time. The within-run and between-run precisions were <12 and <10%, respectively. This analytical method was successfully applied to a study to correlate changes in maternal and placental NFV plasma concentrations in rats following NFV exposure in utero.

Keyword: Nelfinavir

1. Introduction

Nelfinavir (NFV), a potent HIV-1 protease inhibitor, is commonly used in combination with two reverse transcriptase inhibitors in the therapeutic treatment of HIV-infection in pregnant women [1]. The mother-to-child transmission rate has decreased to <2% with the use of combination antiretroviral therapy (ART) [2,3]. Nelfinavir, as with all protease inhibitors, is associated with glucose intolerance, insulin resistance, and new onset of diabetes mellitus [4,5]. Pregnancy is a risk factor for glucose intolerance. Maternal glucose intolerance of gestational diabetes is associated with a high incidence of maternal–fetal complications during pregnancy [6]. The placentas recovered from gestationally diabetic women show greater prevalence of hypertrophy and increases in mass, size, weight, surface area [7,8]. It remains unknown whether the use of NFV exacerbates the risk for gestational diabetes mellitus, and whether a dose- or concentration-dependent relationship exists.

Nelfinavir forms two major metabolites, 3′-methoxy-4′-hydroxynelfinavir (M1) and hydroxyl-*t*-butylamidenelfinavir (M8), following oral administration [9]. Nelfinavir is predominantly metabolized to the M1 form in rats (personal communication with Dr. Ellen T. Wu, Agouron Pharmaceuticals Inc., La Jolla, CA). Numerous reversed-phase high-performance liquid chromatography (HPLC) methods have been published for the quantitative determination of NFV [10–13]. These methods have proved to be unsuccessful in separating NFV from its M1 metabolite by our laboratory. Several liquid chromatography–tandem mass spectrometry (LC/MS/MS) methods have been published for simultaneous determination of five or more HIV-1 protease inhibitors, and the M8 metabolite of NFV [9,14,15]. To our knowledge, only one published LC/MS/MS assay has been developed for simultaneous quantification of NFV and its two metabolites, M1 and M8 [9]. This method was validated for NFV over the concentration ranges of 20–3000 ng/mL, and 1–1000 ng/mL for NFV and M1, respectively. In addition, the sample preparation consisted of

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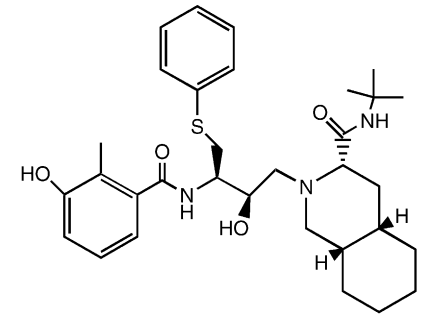
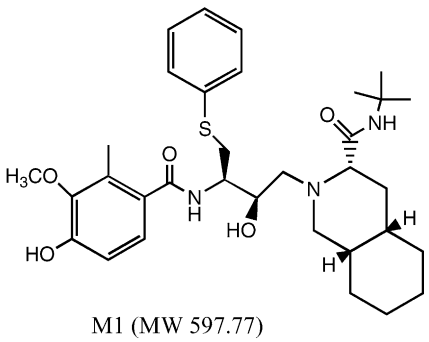
extraction with a mixture of ethyl acetate and acetonitrile (ACN), evaporation, and reconstitution with the mobile phase [9].

Although the LC/MS/MS methods provide improved sensitivity for NFV as compared to the HPLC method [9,10–15], there is still a need to increase the sensitivity for quantitative determination of NFV during pregnancy. Pregnancy is associated with numerous physiologic changes that include increases in plasma volume, fat stores, cardiac output, glomerular filtration, hepatic blood flow, and altered protein binding [16,17]. These physiologic changes may lead to lower levels of NFV during pregnancy. Recently, a patient with HIV-infection who developed virological breakthrough while receiving a NFV-containing regimen was reported [18]. Nelfinavir plasma levels decreased from 7.03 μg/mL during nonpregnant state to 2.23 μg/mL at 36 weeks gestation. The decreases in plasma peak concentrations, area under the curve, half-life and clearance suggest that pregnancy may have affected pharmacokinetic disposition of NFV, resulting in virologic breakthrough and development of drug resistance in the patient [18]. In a separate study, 11 of 14 patients had undetectable plasma levels of NFV at time of delivery [19]. The HPLC assay used for this study had a lower limit of quantitation for NFV at 250 ng/mL [11].

The major aim of this investigation was to develop a simple and highly sensitive LC/MS/MS method for the detection of NFV and its M1 metabolite in dams at day 20 of gestation following exposure to NFV in utero. The structures of NFV and M1 are shown in Fig. 1. Here we combined an on-line column-switch method with LC/MS/MS to further increase the sensitivity, as well as reducing sample preparation time by eliminating the need for evaporation by nitrogen and sample reconstitution. This method was applied in a study to correlate maternal NFV concentrations with glucose levels, and placental changes following in utero exposure in rats.



Nelfinavir (MW 567.78)



M1 (MW 597.77)

Fig. 1. Structures of nelfinavir and its M1 metabolite.

2. Experimental

2.1. Materials

Nelfinavir and M1 were kindly provided by Agouron Pharmaceuticals Inc. The internal standard reserpine was obtained from Sigma (St. Louis, MO). Female and male Sprague–Dawley rats were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN), HPLC grade ACN, methanol, and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA). Drug free heparinized rat plasma used was obtained from Harlan Bioproducts for Science (Indianapolis, IN).

2.2. Animals

All rats were housed in the animal facility in house and cared for in accordance with the Ohio State University (OSU) Institutional Animal Care and Use guidelines. A total of 22 female Sprague–Dawley rats were randomly assigned to a control, low-dose (100 mg/kg/day), or high-dose NFV-treated (400 mg/kg/day) group. Treatment was initiated 1 week prior to mating. Rats were mated overnight within a 12 h period once weekly. Vaginal smears were conducted with sperm-positive smears denoting day 0 of pregnancy. Carboxymethylcellulose suspension was administered to the control rats in identical volumes per weight as in the NFV-treated groups. Necropsy was performed on all females at day 20 of gestation. Maternal blood samples were collected at the time of necropsy at 20 ± 1.7 h after last dosing. The uterus and its contents were immersed in PBS following exsanguination. The placentas and fetuses were isolated, weighed and measured using a caliper. Placental surface area was calculated using the equation: $\pi(a/2)(b/2)$. Nonfasting glucose concentrations of maternal serum were analyzed by YSI 2500 Biochemistry Select Analyzer (Yellow Spring, OH). The OSU Institutional Animal Care and Use Committee approved the study protocol.

2.3. Instrumentation

A PE Sciex API 300 triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) coupled to a Shimadzu HPLC system consisting of a column-switch device (VICI, Valco Instruments Inc., Houston, TX), two high-pressure solvent delivery pumps (LC-10AD, Shimadzu, Columbia, MD), a Shimadzu autosampler (SIL-10A) and a system controller (SCL-10A). Samples were first loaded into an extraction col-

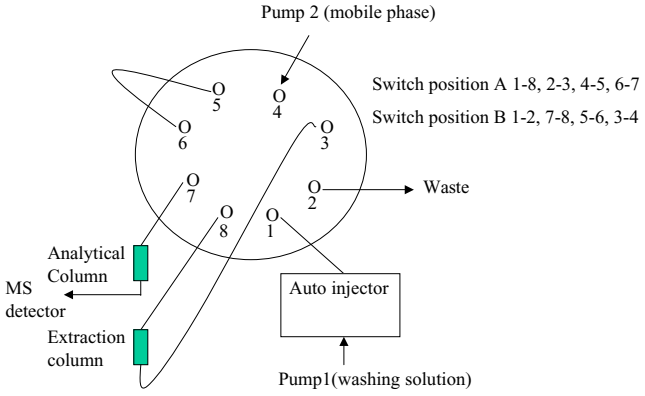


Fig. 2. Schematics of the on-line column-switch system.

umn (MetaSil Basic 2 × 10 mm, 30 μm C8, Metachem, Torrance, CA) via an auto injector by pump 1 when the switch was set in the A position. The loading solvent, composed of 95:5 methanol/water was pumped through pump 1 at the rate of 1 mL/min for 1 min to wash out water soluble impurities from the extraction column, while pump 2 delivered the chromatographic mobile phase (60:40 ACN/0.05% acetic acid) through the guard column (Betabasic 8, 2 × 10 mm, Keystone Scientific, Bellefonte, PA) then to the analytical column (Javelin 2 × 20 mm C8, 5 μm, Keystone Scientific). After 1 min, the switch was changed to the B position, then pump 2 backflushed the analytes from the extraction column to the Guard-Analytical (G-A) columns with the mobile phase. The column-switch device is shown in Fig. 2.

Mass spectrometry parameters used were: electrospray ionization with ionspray voltage of +4500 V was selected, and orifice voltage (OR) was optimized at 15 V. Mass calibration was performed on both the first and the third quadrupoles, using a solution of polypropylene Glycol (PPG) in 3 mM ammonium acetate. Three multiple reaction monitor (MRM) channels were set at 568.4–330, 609.2–397, and 598.2–360, for monitoring NFV, reserpine, and M1, respectively. No MRM cross-talk between NFV and M1 was observed, i.e. NFV did not give a daughter at *m*/*z* 360 and M1 did not yield a daughter ion at *m*/*z* 330.

2.4. Preparation of standards and controls

The stock solutions of NFV and M1 were made by dissolving the appropriate compounds with 50:50 ACN/water to make a concentration each 1 mg/mL. The stock solutions were appropriately diluted with ACN/water to various concentrations for use as working standards. Ten microliters each of NFV and M1 working standard solutions with proper concentrations were spiked into 0.1 mL drug-free rat plasma to give a range of concentrations from 0.8 to 400 ng/mL for NFV, and 0.2 to 80 ng/mL for M1, respectively. Quality control (QC) samples were prepared at three different concentrations of each analyte using a prepared stock solution. The internal standard (IS) solution reserpine was prepared with 50:50 ACN/water at a concentration of 1 mg/mL and the solution was further diluted to 4 μg/mL for use as working solution. A 10 μL of IS working solution was added to appropriate samples in 1.5 mL microcentrifuge tubes and the content was mixed gently. Then to each tube was added 300 μL cold (−20 °C) ACN and the content was mixed by vortex for 20 s. The tubes were centrifuged at 15 900 × *g* for 10 min. A 50 μL aliquot was injected into the on-line column-switching system for analysis. Because this is an on-column method, only the overall recoveries of NFV (4 and 400 ng/mL), M1 (0.8 and 80 ng/mL), and IS (100 ng/mL) were assessed, and they were accomplished by comparing the peak areas of analytes obtained from plasma samples to those of drug spiked into the mobile phase.

2.5. Stability

The stability of the analytes and the IS was investigated at various concentrations stored in mobile phase and in rat plasma at 4, 23, and 37 °C. Freshly prepared QC samples, at medium concentration of 40 ng/mL NFV, 8 ng/mL M1, and at high concentration of 400 ng/mL NFV, 80 ng/mL M1, both with 100 ng/mL of IS were incubated in a water bath (Model 180 Series, Precision Scientific, Chicago, IL) at 37 °C for 24 and 72 h. Stability of NFV, M1, and IS in the mobile phase (60:40 ACN/0.05% acetic acid) was also carried out at 4 and 23 °C for 24 h. Three replicates were used for each solution at each of the storage conditions. The concentrations of the analytes were compared to the mean of back-calculated values of the samples that were freshly prepared. The freeze-thaw stability (at two concentrations and three cycles) of NFV and M1 were evaluated and the mean overall changes was <10%.

2.6. Statistical analysis

The maternal glucose and placental data obtained from the rats are presented as arithmetic mean ± standard deviation (S.D.). Differences between groups were determined by analysis of variance, post hoc testing by the method of Tukey. The effects of treatment dose on placenta:fetal weight ratio, and placental surface area were tested for group differences by two different methods. First, the average placenta: fetal weight ratio and placental surface area were calculated for each mother, and comparisons were made using nonparametric Wilcoxon rank sum tests. Next, a correlated data models, using SAS' PROC MIXED, were applied in order to account for the correlations among pups from the same mother. Pearson correlation coefficients were computed to ascertain the linearity between NFV levels, data were combined from both groups, and maternal glucose levels, average placenta:fetal weight ratio, and average placenta surface area. A *P*-value of ≤0.05 was considered significant.

3. Discussion and conclusion

3.1. Method validation

Extracted ion chromatograms from drug-free plasma and from a standard sample spiked with 0.8 ng/mL of NFV, 0.2 ng/mL of M1, and 100 ng/mL of IS are shown in Fig. 3. The retention times of NFV, M1, and IS were 2.6, 2.6, and 2.4 min, respectively. NFV and M1 were co-eluted under the chromatography conditions, but were resolved by their respective masses with selected MRM channels.

Linearity was observed for NFV in the concentration range between 0.8 and 400 ng/mL and for M1 in concentration range between 0.2 and 80 ng/mL, both with a regression coefficient of >0.99. The limit of quantitation (LOQ), defined as a signal to noise ratio of 5, was determined as 0.8 ± 0.09 and 0.4 ± 0.04 ng/mL for NFV and M1, respectively, using 0.1 mL rat plasma. The LOQ of NFV is significantly lower than those recently reported LC/MS/MS [9,14,15]. Chi et al. [15] reported a LOQ of 5 ng/mL and Crommentuyn et al. [14] reported a LOQ of 50 ng/mL for NFV. The use of the column-switch system in the current method may contributed the improved LOQ, since removal of column-switch and extraction column generated poor NFV signals with wider peak and lower peak area tested by pure standard solutions.

The within-run and between-run characteristics for NFV and M1 at three concentrations levels are shown in Table 1. The within-run coefficients of variation (CVs) were 11.8, 10.3, 9.8% at 0.8, 40, 400 ng/mL for NFV, and 12.1, 9.8, 10.0% at 0.4, 8.0, 80.0 ng/mL for M1, respectively ($n = 6$). The between-run CVs and accuracy values are also listed in Table 1. As shown, the between-run CVs were less than 8.8% for NFV and 8.7% for M1, and accuracy values ranged from 97.9 to 104.2% for NFV and from 95.0 to 103.4% for M1.

Recovery data of NFV, M1, and the IS are listed in Table 2. The mean recovery value for NFV is close to 100%, indicating that the matrix effect for NFV was negligible. The mean recovery values for M1 ranged from 101.3 to 113.1%, possibly due to some ion enhancement effect of the matrix. The recovery for the IS at 100 ng/mL was 76%, indicating some ion suppression existed for reserpine. The standard deviation values for all three compounds were less than 6.3%, indicating that the column switching method is highly reproducible and with constant recovery values.

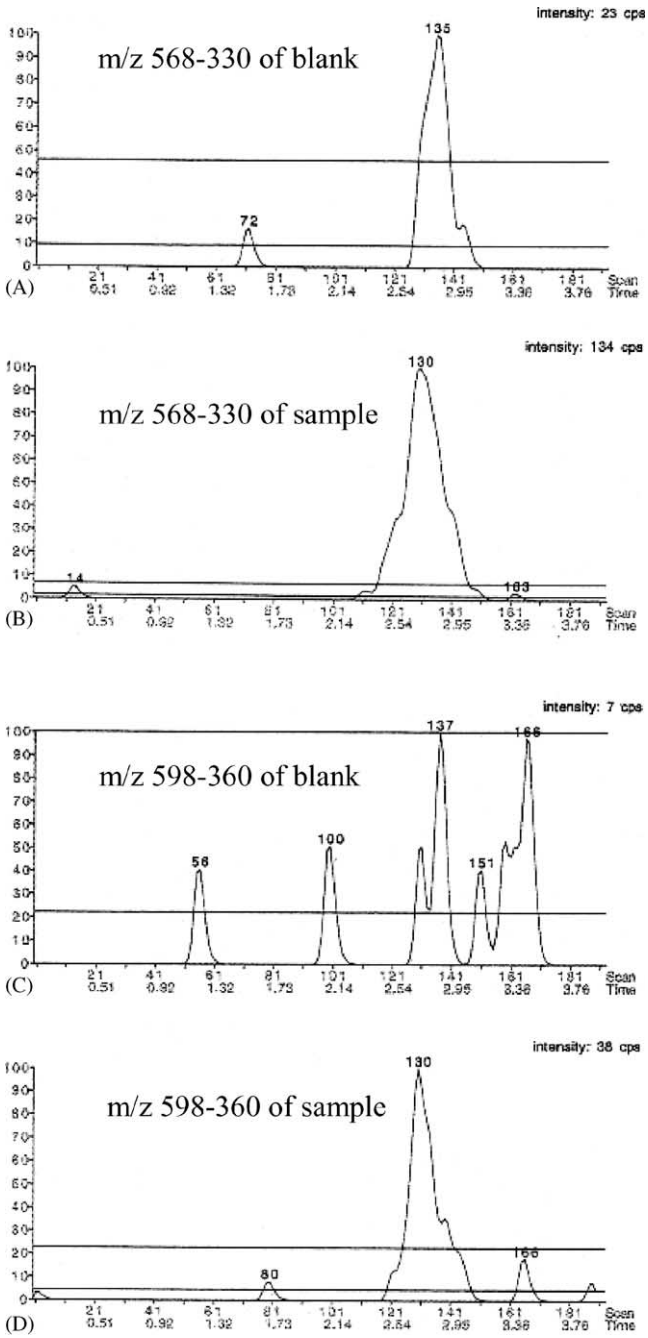


Fig. 3. Extracted ion chromatograms from blank rat plasma and a rat plasma sample spiked with 0.8 ng/mL of NFV, 0.2 ng/mL M1, and 100 ng/mL of internal standard. MRM chromatograms are shown in (A) NFV blank with MRM set at *m/z* 568.4–330; (B) NFV standard at *m/z* 568.4–330, with the signal to noise (S/N) ratio of >5; (C) M1 blank at *m/z* 598.2–360; (D) M1 sample at *m/z* 598.4–360 with the S/N of >5. The smooth factor was 5.

3.2. Column switch assembly

There are merits of column-switch approach in sample treatment when compared to other approaches such as solid-phase extraction (SPE) and liquid–liquid (L–L) extraction [20,21]. This system was on-line and fully automated; the total run time was only 5 min per sample following simple protein precipitation. There was no evaporation and reconstitution needed as in the case of off-line SPE or L–L methods. Using the column switch method, the analytes were enriched in the extraction column, then back flushed into analytical column, and resulting in higher sensitivity as compared to other reported methods [9,14]. No appreciable

Table 1
Method validation of (a) nelfinavir and (b) M1 in 0.1 mL rat plasma

	Concentration (ng/mL in plasma)			
	0.8	4	40	400
(a)				
Within-run number				
1	0.77	4.6	44.98	371.8
2	0.71	4.59	47.37	421.5
3	0.89	4.86	45	406.2
4	0.83	4.89	46.4	423
5	0.93	3.75	36.4	448.6
6	0.7	3.88	38.95	496.7
Average	0.81	4.43	43.18	428.0
S.D.	0.09	0.49	4.43	42.06
CV (%)	11.8	11.1	10.3	9.8
Accuracy	100.6	110.7	108.0	107.0
Between-run number				
1	0.79	3.75	36.48	393.4
2	0.74	3.84	37.2	417
3	0.77	4.61	44.98	393.4
4	0.73	4.21	41.3	395.8
5	0.89	4.02	36.78	486.4
6	0.78	4.58	38.3	406.2
Average	0.78	4.17	39.17	415.4
S.D.	0.06	0.37	3.34	36.00
CV (%)	7.3	8.8	8.5	8.7
Accuracy	97.9	104.2	97.9	103.8
	Concentration (ng/mL plasma)			
	0.4	2	8	80
(b)				
Within-run number				
1	0.42	2.1	9.08	73
2	0.4	1.98	9.43	82.3
3	0.34	1.77	9.32	81.6
4	0.44	1.93	9.2	77
5	0.35	2.02	7.4	93.2
6	0.33	2.05	7.9	93.4
Average	0.38	1.98	8.72	83.42
S.D.	0.05	0.12	0.85	8.36
CV (%)	12.1	5.9	9.8	10.0
Accuracy	95.0	98.8	109.0	104.3
Between-run number				
1	0.41	1.97	7.38	81.1
2	0.39	1.95	8.2	80.3
3	0.36	2.08	9.08	79.2
4	0.34	1.94	9.1	93.2
5	0.42	2.05	7.62	77
6	0.41	2.13	8.23	93.3
Average	0.39	2.02	8.27	84.02
S.D.	0.03	0.08	0.72	7.28
CV (%)	8.2	3.9	8.7	8.7
Accuracy	97.1	101.0	103.4	105.0

loss of analytes was found with 5% methanol in water at the flow rate of 1 mL/min as loading and washing solvent. Higher organic solvent may introduce protein precipitation in extraction column and may cause the loss of analytes from the extraction column.

Table 2
Column switching recovery for nelfinavir, M1, and internal standard ($n = 5$)

Compound	Norminal concentration (ng/mL)	Recovery (%)	S.D. (%)
NFV	4	102.4	6.3
	400	97.2	3.9
M1	0.8	101.3	5.8
	80	113.1	4.6
Reserpine	100	75.7	2.3

3.3. Stability

Nelfinavir was previously reported by Crommentuyn et al. [14] as stable in the plasma and in the HPLC mobile phase solution. We reinvestigated the stability of NFV with lower concentration solutions and incorporated the stability study of M1 metabolite, and reserpine. The stability values are listed in Tables 3 and 4. As shown, all these compounds were found to be stable and degraded less than 10% under all investigated conditions. Stability data for M1 were not available previously, while stability data for NFV consisted with the reported results [14,15].

3.4. Application

Necropsy was performed on eight control (total of 95 concepti), seven low-dose (total of 99 concepti) and seven high-dose NFV-treated (total of 90 concepti) dams. The mean (±S.D.) placenta:fetal weight ratios were 0.138 ± 0.018, 0.157 ± 0.022, and 0.156 ± 0.027 g in the control, low-dose and high-dose NFV-treated groups, respectively ($P < 0.001$). The placental surface area was significantly larger in the low-dose and high-dose NFV-treated groups as compared to control, $1.42 \pm 0.142\,cm^2$, $1.51 \pm 0.137\,cm^2$ versus $1.49 \pm 0.181\,cm^2$, respectively ($P < 0.05$). No statis-

Table 3
Stability data for NFV and M1 in rat plasma ($n = 3$)

	Nominal mean concentration	Measured mean concentration	S.D.	CV (%)	Accuracy (%)
NFV					
24 h at 37 °C	40.6	43.7	1.9	4.4	107.6
	398.6	417.2	21.1	5.1	104.7
72 h at 37 °C	40.6	43.8	3.6	8.1	107.9
	398.6	382	30.8	8.1	95.8
M1					
24 h at 37 °C	8.2	8.5	0.7	8.1	103.7
	79.1	87	6.1	7.0	110.0
72 h at 37 °C	8.2	7.52	0.7	9.3	91.7
	79.1	73.1	6.8	9.3	92.4
Reserpine					
24 h at 37 °C	100	92.1	9.1	9.9	92.1
72 h at 37 °C	100	94.9	6.2	6.5	94.9

Table 4
Stability data for NFV and M1 in 60:40 acetonitrile/0.05% HAc ($n = 3$)

	Nominal mean concentration	Measured mean concentration	S.D.	CV (%)	Accuracy (%)
NFV					
24 h at 4 °C	1.9	1.8	0.2	11.1	94.7
	40.4	43.7	1.9	4.4	108.2
	408	388.2	28.1	7.2	95.1
24 h at 23 °C	1.9	1.7	0.2	11.8	89.5
	40.4	43.8	3.6	8.2	108.4
	408	382	25.4	6.7	93.6
M1					
24 h at 4 °C	0.42	0.46	0.05	10.9	109.5
	7.5	8.3	0.7	8.4	110.7
	81.8	77	5.2	6.8	94.1
24 h at 23 °C	0.42	0.38	0.03	7.9	90.5
	7.5	8.4	0.4	4.8	112.0
	81.8	77.9	4.3	5.5	95.2
Reserpine					
24 h at 4 °C	100	95.1	9.2	9.7	95.1
24 h at 23 °C	100	93.2	7.6	8.2	93.2

Table 5
Maternal glucose and placental outcomes in rats following in utero exposure to nelfinavir

Parameters	Control ($n = 95$ concepti)	Low-dose NFV[a] ($n = 99$ concepti)	High-dose NFV[b] ($n = 90$ concepti)	P-value
Maternal glucose[c] (mg/dL)	100.7	114.0	116.6	NS
Placenta:fetal weight ratio (g)	0.138 ± 0.018	0.157 ± 0.022	0.156 ± 0.027	<0.001
Placental surface area (cm^2)	1.42 ± 0.142	1.51 ± 0.137	1.49 ± 0.181	<0.05

[a] Low-dose NFV: 100 mg/(kg day).
[b] High-dose NFV: 400 mg/(kg day).
[c] Normal glucose range in rats: 45–107 mg/dL.

tical difference was noted for maternal glucose levels among the groups; however, there was a trend for higher glucose levels in the two treatment groups (Table 5). The observed placental changes are consistent with the clinical findings of gestational diabetes.

We applied the described method to determine if there is an association between plasma levels of NFV and maternal glucose, and placental outcomes, following in utero exposure to NFV. Plasma levels of NFV and the M1 metabolite are shown in Table 6. The mean maternal plasma levels of NFV and M1 were 4.9 ± 2.7 and 1.4 ± 0.8 ng/mL in the low dose treated group, and 6.8 ± 4.4 and 1.1 ± 0.7 ng/mL in the high-dose treated group, respectively. No significant differences were noted for maternal glucose, placental:fetal weight ratio, and placental surface area between the two treatment groups by both nonparametric Wilcoxon rank sum tests and correlated data models. Similarly, there were no significant relationships between NFV levels with maternal glucose, and placental changes. Our data suggest that the observed placental changes and maternal glucose do not appear to be dose-dependent or concentration-dependent.

A rapid, accurate, sensitive, and specific on-line column-switch LC/MS/MS method was developed and validated for determining NFV and M1 in plasma from rats. The advan-

Table 6
Nelfinavir and M1 in rat plasma samples

Sample ID	NFV (ng/mL)	M1 (ng/mL)
Low dose rat: NFV 100 mg/(kg day)		
L1	4.55	1.55
L3	3.35	0.7
L4	8.3	2.9
L5	2.65	0.7
L7	8	1.7
L8	6.05	1.6
L9	1.2	0.5
Low dose average	4.9	1.4
S.D.	2.7	0.8
High dose rat: NFV 400 mg/(kg day)		
H2	5.15	0.8
H4	7.35	1.25
H5	6.8	1.05
H6	2.8	0.7
H8	10.15	1.75
H9	14.05	2.3
H10	1.3	0.15
High dose average	6.8	1.1
S.D.	4.3	0.7

Note: All data are the average of two measurements.

tages of this method include rapid and simple sample preparation, high selectivity and sensitivity, and improved LOQ for each analyte. This assay is currently been used to analyze placental and fetal liver tissues from rats following NFV exposure in utero. In addition, its application to analyze plasma and cord blood samples from HIV-1 infected pregnant women is explored.

Acknowledgements

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