Determination of Nevirapine Using HPLC with UV Detection

**INTRODUCTION**

Combination therapy has proven to be one of the most effective approaches to treat HIV infection. Nevirapine is a non-nucleoside reverse transcriptase inhibitor (NNRTI) with activity against human immunodeficiency virus type 1 (HIV-1) that is already marketed for the treatment of HIV-1 infected adults. Nevirapine is recommended for treating HIV infections in combination with other reverse transcriptase inhibitors such as stavudine and lamivudine.

The method in the United States Pharmacopeia (USP)—monograph for determining nevirapine and its related compounds, A and B—uses a reversed-phase separation with UV detection. The method calls for a 4.6 × 150 mm column packed with L60 (spherical, porous silica gel, 10 µm or less in diameter, the surface of which has been covalently modified with alkyl amide groups and endcapped). Due to the strong retention of impurity C, the separation requires about 30 min. Here, we report an optimized HPLC-UV method that requires less time per analysis and satisfies the chromatographic parameters of the USP method.

**EQUIPMENT**

Dionex UltiMate® 3000 Intelligent LC system:
- LPG-3000 pump
- WPS-3000 autosampler
- TCC 3200 column compartment
- VWD-3400 detector

Chromeleon® 6.70 chromatography management system

**REAGENTS AND STANDARDS**

Acetonitrile, Fisher HPLC Grade or equivalent
Water, Milli-Q water from Milli-Q Gradient A10
Ammonium phosphate monobasic (NH₄H₂PO₄), Fluka ACS reagent, ≥99%, or equivalent
Nevirapine (99.99%), stavudine (98.19%), and lamivudine (99.43%) standards, generous gifts from a customer
Thymine, 99% from Sigma

**PREPARATION OF MOBILE PHASE AND STANDARDS**

To prepare the mobile phase, weigh 2.882 g NH₄H₂PO₄ into a 200-mL beaker. After dissolving with water, move the solution to a 1000-mL volumetric flask and dilute to 1000 mL. Filter through a 0.45-µm PVDF Millicup-HV filter.
Prepare the stock standard solution by weighing 100 mg of nevirapine into a 250-mL volumetric flask together with 50 mL of MeCN and 50 mL of 25 mM NH$_4$H$_2$PO$_4$ buffer. After sonication for 5 min, add 90 mL water and continue sonication for 10 min. After cooling, bring the solution to volume with water and filter an aliquot through a 0.2-µm filter. The concentration of nevirapine was 0.4 mg/mL.

Prepare serial standard solutions with concentrations of 0.01, 0.05, 0.10, and 0.30 mg/mL nevirapine by taking the proper amount of stock standard solution and diluting with a mixture of 25 mM ammonium phosphate and acetonitrile that equal the initial eluent concentration. To prepare 100 mL of this mixture, add 18 mL of acetonitrile to 82 mL of the 25 mM ammonium phosphate solution.

**PREPARATION OF SAMPLES**

A nevirapine sample solution was a generous gift from a customer with a labeled concentration of 0.24 mg/mL nevirapine, 0.00012 mg/mL nevirapine-related compound A, and 0.00012 mg/mL nevirapine-related compound B.

Dilute the sample 1:4 and 1:9 with mobile phase at its initial concentration (see Preparation of Standards for preparation of this mobile phase concentration).

**CHROMATOGRAPHIC CONDITIONS**

| Column: Acclaim® PA, 4.6 × 150 mm, 5 µm (P/N 061320) | Mobile Phase: 25 mM NH$_4$H$_2$PO$_4$ and MeCN (see gradient table) |
| Flow Rate: 1.5 mL/min | Inj. Volume: 20 µL |
| Detection: Absorbance at 220 nm |
| Column Temperature: 35 ºC |

**Gradient Table**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>25 mM NH$_4$H$_2$PO$_4$ (%)</th>
<th>MeCN (%)</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>82</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>70</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>10.0</td>
<td>70</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>10.5</td>
<td>82</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>15.0</td>
<td>82</td>
<td>18</td>
<td>5</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

**Separation of Nevirapine and Its Related Compounds A and B on the Acclaim Polar Advantage (PA) Column**

The chemical structure of nevirapine is shown in Figure 1. Using isocratic conditions, nevirapine and its related compounds A and B could be baseline resolved, but the chromatographic resolution between nevirapine and its related compounds were not as high as the values prescribed in the USP method. Therefore, we developed a gradient method.

The Acclaim PA column contains a sulfonamide-embedded reversed-phase silica-based stationary phase ideal for separating nevirapine and its related compounds, A and B. This stationary phase shares some chemical properties with L60. It also has selectivity similar to an ordinary C18 column for many analytes of low polarity, and is compatible with aqueous-only mobile phases for analytes of high polarity. Using the Acclaim PA under the chromatographic conditions (eluents, flow rate, detection wavelength, column dimensions, and column temperature) described in the USP monograph method for nevirapine, we developed a gradient separation of nevirapine and its related compounds A, B, and C. This separation meets the chromatographic requirements of the USP method. Using the Acclaim PA, related compound C is eluted within 11 min, allowing a total analysis time about half that of the USP method.

![Figure 1. Chemical structure of nevirapine.](24305)
Resolution

Figure 2 shows a chromatogram of the undiluted nevirapine sample. The calculated resolution between nevirapine-related compound B and nevirapine was 6.5, and that between nevirapine and nevirapine-related compound A was 10.9, exceeding the values in the USP method. The USP values are ≥ 5.0 and ≥ 7.4, respectively.

Linearity

Calibration linearity for UV detection of nevirapine was found to extend over the range from 5.0 mg/mL to 300 mg/mL based on making replicate injections (n = 6) of serial standard solutions of nevirapine at four concentrations. The linear regression equation was:

\[ y = 1095.6x \]

where y is peak area (mAU·min), x is sample concentration (mg/mL), and the origin was used as the first point. Figure 3 shows the linearity of nevirapine (correlation coefficient, R^2, of 0.9999). Table 1 summarizes the related data. The detection limit of nevirapine, calculated by using S/N = 3, was 3.18 ng/mL.

Reproducibility

The reproducibility was estimated by making replicate injections (n = 6) of a nevirapine standard solution (0.05 mg/mL). The relative standard deviation (RSD) was 0.030% for retention time, 0.284% for peak area, and 0.366% for peak height.

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**Figure 2. Chromatogram of nevirapine and its related compounds.**

**Table 1. Data of Nevirapine Standards for Calibration**

<table>
<thead>
<tr>
<th>Injection Number</th>
<th>0.01 (mg/mL)</th>
<th>0.05 (mg/mL)</th>
<th>0.1 (mg/mL)</th>
<th>0.3 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.234</td>
<td>54.198</td>
<td>107.71</td>
<td>330.69</td>
</tr>
<tr>
<td>2</td>
<td>11.152</td>
<td>54.457</td>
<td>108.16</td>
<td>330.68</td>
</tr>
<tr>
<td>3</td>
<td>11.129</td>
<td>54.001</td>
<td>107.39</td>
<td>328.70</td>
</tr>
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<td>4</td>
<td>11.108</td>
<td>54.333</td>
<td>107.25</td>
<td>328.05</td>
</tr>
<tr>
<td>5</td>
<td>10.991</td>
<td>54.251</td>
<td>106.96</td>
<td>330.72</td>
</tr>
<tr>
<td>6</td>
<td>10.957</td>
<td>54.179</td>
<td>106.92</td>
<td>328.13</td>
</tr>
<tr>
<td>Average</td>
<td>11.095</td>
<td>54.237</td>
<td>107.40</td>
<td>329.49</td>
</tr>
<tr>
<td>RSD</td>
<td>0.935</td>
<td>0.284</td>
<td>0.442</td>
<td>0.405</td>
</tr>
</tbody>
</table>

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**Figure 3. Linearity of nevirapine (n = 6).**
Sample Analysis

Figure 4 shows the chromatograms of the 1:10 diluted nevirapine sample and that sample spiked with nevirapine. The recovery of nevirapine \( (n = 5) \) ranged from 102% to 102.9%. The average concentration of nevirapine determined in the undiluted sample solution was 0.25 mg/mL, consistent with the labeled value, 0.24 mg/mL.

Application to the Analysis of Other NNRTIs

This method can be used to analyze other non-nucleoside reverse transcriptase inhibitors (NNRTIs) with activity against HIV-1. The commonly used NNRTIs are zidovudine, lamivudine, stavudine, nevirapine, and indinavir. Figure 5 shows the separation of thymine, lamivudine, stavudine, and nevirapine. Indinavir was not analyzed.

Faster Analysis of Nevirapine

As shown in Figure 6, using a 100 mm narrow bore column can shorten the analysis of the nevirapine sample to < 10 min. This analysis should be performed using a high pressure mixing gradient pump to minimize delay volumes and requires a change to a 2.5-µL flow cell.
Performance of the UltiMate 3000 Intelligent LC System
Simultaneous Determination of Nevirapine and Related Compounds with Different Concentrations

As shown in Figure 2, the peak height of the main peak, nevirapine, was 2800 mAU, and the peak heights of the impurities including the related compounds A and B were between 0.3–4 mAU, which demonstrates the exceptional performance of the VWD-3400 detector for simultaneously determining a main constituent and its trace level impurities. This was verified by measuring the linearity of the response of the impurities, related compounds A and B, and nevirapine from replicate injections of the nevirapine sample and 1:4 and 1:9 diluted nevirapine samples, respectively (n = 6). Figure 7 shows an overlay of chromatograms of nevirapine-related compound B at different concentrations, and Figure 8 shows the graphs of peak area versus amount for nevirapine and its related compounds A and B. The correlation coefficients were 0.9999 for both nevirapine and nevirapine-related compound B, and 0.9992 for nevirapine-related compound A, demonstrating the excellent performance of the VWD-3400 over this broad concentration range difference between analytes. Table 2 summarizes related data from this experiment, from which we can conclude that the VWD-3400 detector provides accurate analysis in applications with varying analyte concentrations.

Figure 7. Overlay chromatograms demonstrating trace-level detection of nevirapine-related compound B.

<table>
<thead>
<tr>
<th>Related compound B</th>
<th>Related compound A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_{\text{peak}}$ of Nevirapine (mAU)</td>
<td>$H_{\text{peak}}$ (mAU)</td>
</tr>
<tr>
<td>2820</td>
<td></td>
</tr>
<tr>
<td>Original sample</td>
<td>3.6</td>
</tr>
<tr>
<td>1:4 diluted sample</td>
<td>0.7</td>
</tr>
<tr>
<td>1:9 diluted sample</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* Mean value of six determinations
Carry-Over Performance of the WPS-3000

Figure 9 shows exceptional carry-over performance for the WPS-3000 autosampler without the need for an external needle wash. There was no cross contamination observed when using WPS-3000 autosampler for this application.

**Figure 8.** Linearity of (A) nevirapine, (B) related compound B, and (C) related compound A \((n = 6)\).

**Fig. 9.** Carry-over test on the WPS-3000 autosampler. Original nevirapine sample solution and blank solution \((25 \text{ mM } \text{NH}_4\text{H}_2\text{PO}_4 \text{ buffer})\) were injected in series.
CONCLUSION

This application note describes an optimized method for determining nevirapine on an UltiMate 3000 Intelligent LC system with an Acclaim PA column. This method meets or exceeds the chromatographic requirements of the USP monograph method for nevirapine while requiring about half the analysis time per sample. This method is optimized on the UltiMate 3000 due to the system’s elimination of cross contamination from the WPS-3000 autosampler, low noise from the VWD-3400 detector, and other benefits. The Acclaim PA and UltiMate 3000 are ideally suited for determining both polar and nonpolar pharmaceutical compounds and their impurities.

PRECAUTIONS

Exercise care when handling acetonitrile, ideally filling the eluent bottle in a fume hood. Use proper methods for disposal of waste.

REFERENCES