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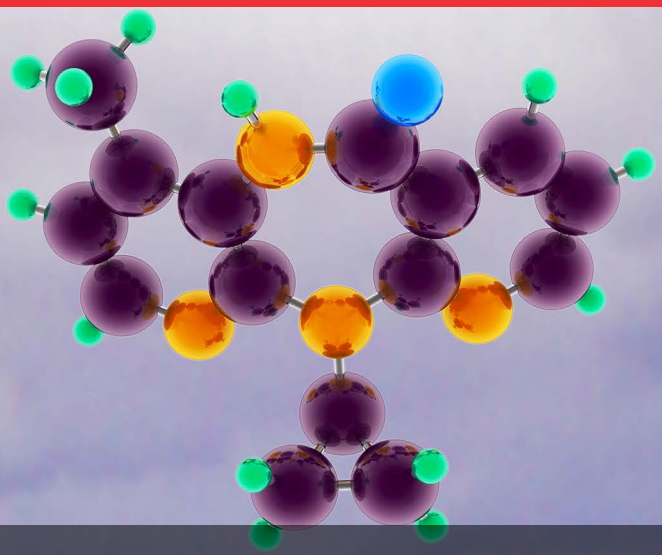

APPLICATION NOTE 72603

Simultaneous quantification of nevirapine and low-level impurities

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Keywords
Thermo Scientific Vanquish DAD FG, nevirapine, impurity profiling, linear range

Goal

To demonstrate the wide dynamic range of the new Thermo Scientific Vanquish DAD FG and how it facilitates the quantification of compounds of very different concentrations.

Application benefits

- The new Thermo Scientific™ Vanquish™ DAD FG provides an industry-leading linear range up to 3700 mAU.
- The detector linearity in combination with low baseline noise allows for the simultaneous quantification of nevirapine and its impurities within a single run.

Introduction

In the pharmaceutical industry, product safety and the quality of distributed drugs are of major importance to ensure proper and efficient therapy. However, the pharmacological-toxicological profile of a drug is not only affected by the active pharmaceutical ingredient (API) itself but also by its impurities originating from manufacturing processes or degradation during storage.[1] For that reason, strict regulations on impurity levels that have to be reported, identified, and/or qualified are defined by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).[2,3] Thus analytical monitoring of substances and impurities is a crucial requirement in drug development and production. The challenge for the instrumentation is the absorption difference of high-concentrated API and low-level impurities that need to be reported down to a content of 0.03% of the API.[2]

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Nevirapine is a non-nucleoside reverse transcriptase inhibitor drug that was approved for the antiretroviral therapy of HIV/AIDS patients by the regulatory authorities in the 1990s.[4] For impurity profiling, the United States Pharmacopoeia (USP)[5] provides an HPLC method with UV detection that was transferred into an optimized UHPLC method with ballistic gradient in a previous Thermo Fisher Scientific application brief.[6] This method was used in the current study to demonstrate the capabilities of the new Thermo Scientific Vanquish DAD FG to quantify both the API nevirapine and its impurities A, B, and C in a single run.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N 10001334)
- Fisher Scientific Ammonium acetate, Optima LC/MS (NH_4Ac) (P/N 11317490)
- Fisher Scientific Acetic acid, Optima LC/MS (P/N 10860701)

Certified standards of the following were purchased from reputable vendors:

- Nevirapine
- 11-Ethyl-4-methyl-5,11-dihydro-6H-dipyrido [3,2-b:2',3'-e][1,4]diazepin-6-one (Impurity A)
- 4-Methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4] diazepin-6-one (Impurity B)
- 4-Methyl-11-propyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one (Impurity C)

Sample preparation

Stock solutions of the API nevirapine (1 mg/mL) and the impurities A, B, and C (100–200 µg/mL each) were prepared by dissolving the solids in pure acetonitrile (ACN) and filling up the respective volumetric flasks with mobile phase A (see below). Calibration standards of nevirapine with concentrations of 0.1, 1, 5, 10, 50, 100, 250, 500, 750, and 1000 µg/mL were prepared by dilution of the stock solution with mobile phase A. These standards were injected with a volume of 1 µL. Further calibration levels were emulated by different injection volumes: 600 µg/mL by injection of 0.8 µL of 750 µg/mL standard; 850 µg/mL by injection of 0.85 µL of 1000 µg/mL standard; 1200 µg/mL by injection of 1.2 µL of 1000 µg/mL standard.

A sample that contained 900 µg/mL API and 0.45 µg/mL of each impurity (corresponding to 0.05% of the API) was prepared by mixing of stock solutions and filling up with mobile phase A.

Instrumentation

Thermo Scientific Vanquish Horizon UHPLC system consisting of:

- System Base Vanquish Horizon (P/N VH-S01-A-01)
- Binary Pump H (P/N VH-P10-A-01)
- Split Sampler HT (P/N VH-A10-A-01)
- Column Compartment H (P/N VH-C10-A-01)
- Diode Array Detector FG (P/N VF-D11-A-01)
- Flow Cell Semi-Micro 7 mm, 2.5 µL (P/N 6083.0530)

LC conditions

Column:	Thermo Scientific™ Syncronis™ C18, 2.1 × 100 mm, 1.7 µm, 100 Å (P/N 97102-102130)
Mobile phase:	A: 10 mM NH_4Ac, pH 5.0 with acetic acid/acetonitrile (85/15; v/v) B: Acetonitrile
Flow rate:	0.8 mL/min
Gradient:	0–0.73 min from 30 to 70% B 0.73–1.1 min 70% B 1.1–1.15 min from 70 to 30% B 1.15–2.8 min 30% B
Column temp.:	50 °C (still air mode)
Autosampler temp.:	8 °C
UV detection:	240 nm, 100 Hz, 0.05 s response time, 4 nm bandwidth, wide slit width
Inj. volume:	0.7–1.2 µL; usually 1 µL
Needle wash:	No wash

Data processing and software

Thermo Scientific™ Chromeleon™ 7.2 SR5 Chromatography Data System (CDS) software was used for data acquisition and analysis.

Results and discussion

In the current study, all standards and prepared samples were injected three times. Figure 1 shows a chromatogram of nevirapine and its impurities. Retention times with precision are summarized in Table 1.

In order to discover the linear detection range provided by the new Vanquish DAD FG, the dependence of detector response on the nevirapine amount was recorded over a concentration range from 0.1 µg/mL to 1200 µg/mL. Figure 2 shows the resulting curve. For a linear calibration with permitted offset and no weighting of data points, the regression line of peak heights exhibited a correlation coefficient of 99.965% and a relative standard deviation of less than 3% for standards from 0.1 µg/mL to 850 µg/mL. This corresponds to peak heights of 0.5 mAU to 3700 mAU. Further confirmation for linear behavior in the stated absorbance range was given by comparison of expected and measured detector responses for the following concentration standard. Each successive calibration point was successfully predicted to within an error of less than 5% from the respective preceding data. Thus, the detector provided an excellent linearity for the current application up to 3.7 AU.

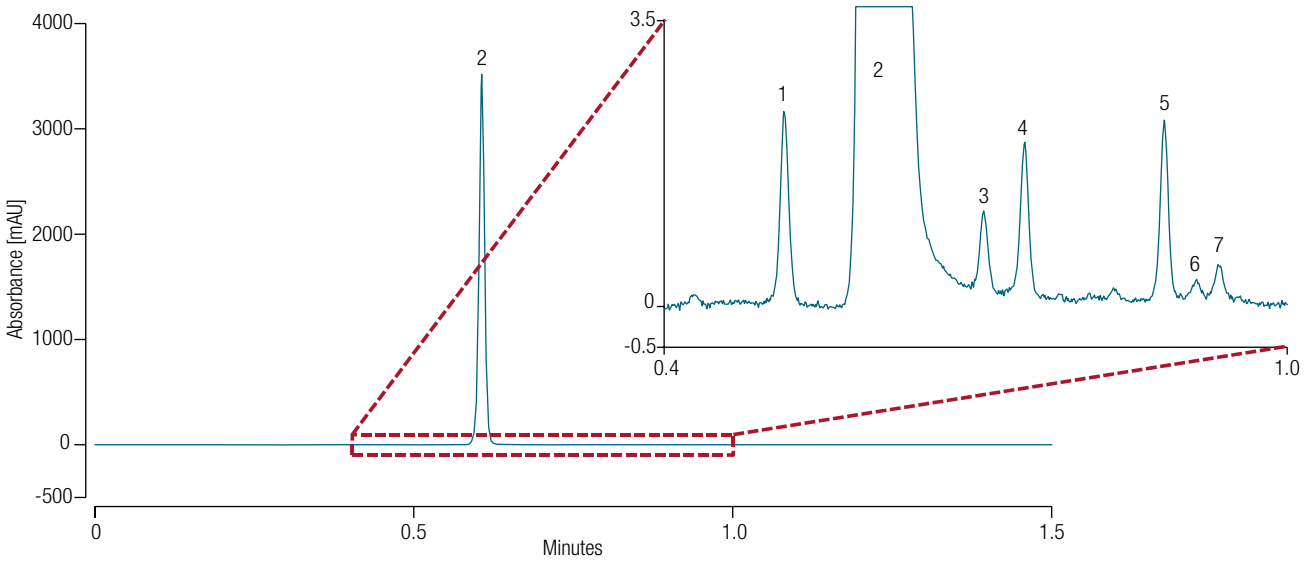

Figure 1. Impurity profile of nevirapine sample (injection volume 0.9 µL) with baseline zoom. For peak assignment see Table 1.

Table 1. Average retention times (t_R) and standard deviations (SD) of nevirapine impurity profiling; determined from all standard and sample injections where the respective peak could be integrated ($18 \leq n \leq 51$).

#Peak	Compound	t_R [min]	t_R SD [min]	t_R RSD [%]
1	Impurity B	0.5160	0.0007	0.14
2	Nevirapine	0.6067	0.0004	0.07
3	Unknown 1	0.7077	0.0005	0.07
4	Impurity A	0.7472	0.0005	0.06
5	Impurity C	0.8815	0.0005	0.05
6	Unknown 2	0.9116	0.0014	0.15
7	Unknown 3	0.9346	0.0008	0.09

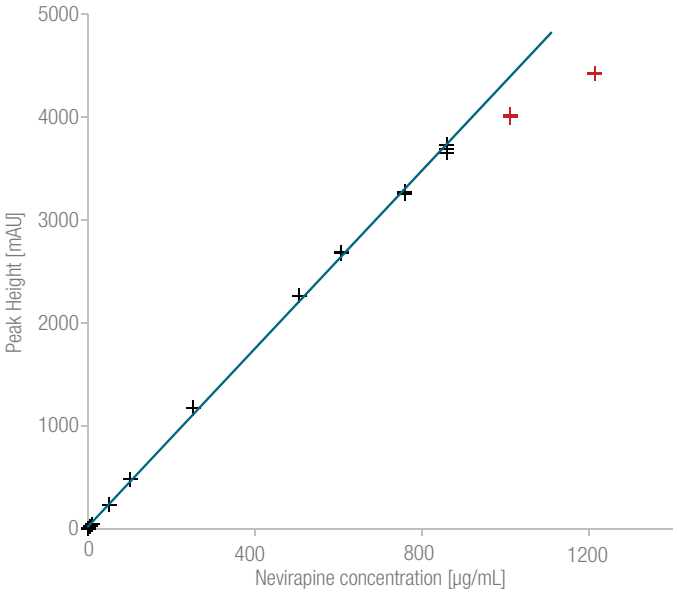

Figure 2. Concentration vs. peak height plot of nevirapine with data points that were considered for calibration (black) and data points that were eliminated from calibration due to curve decline (red). Linear calibration with permitted offset and no weighting.

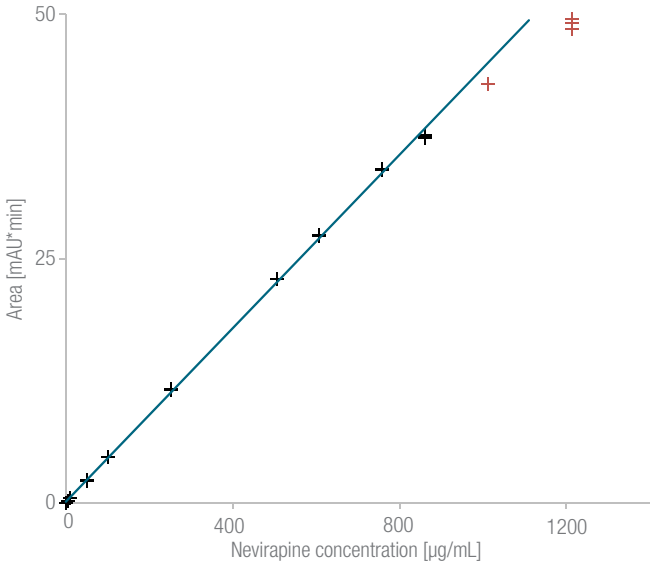

Figure 3. Concentration vs. peak area plot of nevirapine with data points that were considered for calibration (black) and data points that were eliminated from calibration due to curve decline (red). Linear calibration with permitted offset and weighting of calibration points by 1/amount.

This linearity supports the quantification of peaks of extremely different concentrations, eliminating the need for the preparation and injection of different dilutions. If responses are equivalent, this is also valid for the quantitative estimation of several compounds by calibrating with just one compound. This is common practice in pharmaceutical impurity profiling since impurities usually are structurally related to the API. Depending on the drug development stage, impurities may not be fully characterized and therefore are unavailable as reference material. For nevirapine profiling, we applied this procedure by quantifying API and impurities based on the nevirapine calibration curve. For this purpose, we did not utilize the calibration based on peak height that was shown in Figure 2. Instead, we calculated a calibration curve based on peak area with permitted offset and weighting of calibration points by 1/amount (Figure 3). This weighting is easily done in Chromeleon CDS software and cancels out the excessive influence of higher concentration points on the calibration curve. With this approach, low and high concentrations almost equally affect the curve and quantification of peaks over the whole range is improved. Over the same concentration range as before (0.1–850 µg/mL), a correlation coefficient of 99.984% was obtained and deviations of expected and measured response were less than 5% (procedure as described earlier).

For nevirapine, a maximum daily dose of 400 mg translates into a reporting threshold of impurities in new drug substances of 0.05% given by the ICH.[2] In the pure nevirapine standard (850 µg/mL), all six aforementioned impurities were detectable but exhibited relative areas of 0.006% to 0.025%. This was far below the threshold of 0.05% and peaks were not quantifiable due to signal-to-noise ratios (S/N) of less than 10 except for Unknown 1 and Unknown 3. Because of this, a nevirapine sample spiked with 0.05% of impurities A, B, and C related to the API was prepared. The corresponding chromatogram is shown in Figure 1 and the quantitative results are listed in Table 2. The measured amount of the API deviates less than 2% from the expected amount. For the spiked impurities, this deviation is between 6% and 21%, reflecting excellent results under the consideration that equal responses of all compounds are just an approximated assumption and spiked impurity quantities are also affected by inherent concentrations below the quantification limit. Based on the highest and lowest standard that fit the calibration curve, a quantification down to 0.012% relative area is possible with the presented method.

Table 2. Average results of nevirapine and impurity quantification from calibration curve in Figure 3 and three injections of spiked nevirapine sample (see Figure 1). Numbers in red are not reliable as areas were smaller than the lowest calibration standard and/or S/N were less than 10.

#Peak	Compound	Area [mAU*min]	Relative Area [%]	S/N	Determined Amount [µg/mL]	Spiked Amount [µg/mL]
1	Impurity B	0.0225	0.063	70	0.49	0.405
2	Nevirapine	35.7293	99.791	137291	797.0	810
3	Unknown 1	0.0088	0.025	23	0.19	-
4	Impurity A	0.0175	0.049	43	0.38	0.405
5	Impurity C	0.0205	0.057	75	0.45	0.405
6	Unknown 2	0.0019	0.005	8.5	0.04	-
7	Unknown 3	0.0036	0.010	12	0.07	-

Conclusion

- The new Vanquish DAD FG combines a very wide linear range with the best noise performance, enabling for the simultaneous quantification of APIs and impurities within a single run.
- Excellent quantitative results were obtained for the API nevirapine and its impurities with an optimized UHPLC method with deviations from expected amounts of less than 2% for the API and 6–21% for impurities under the approximated assumption of equivalent responses. Impurity quantification was possible down to 0.012% relative area if the linear detection range is fully exploited.

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