

Quantification of paclitaxel, its degradants, and related substances using UHPLC with charged aerosol detection

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Application benefits

- Uniform response in gradient elution using charged aerosol detection with the Thermo Scientific™ Vanquish™ Flex Inverse Gradient LC systems
- Quantification of multiple API-related species by a single calibrant

Goal

First, to demonstrate the ability to quantify multiple impurities with a single calibrant by using the inherent uniform response of charged aerosol detection (CAD). Second, to highlight the capabilities of the Thermo Scientific™ Vanquish™ Flex Inverse Gradient LC system to provide inverse gradient compensation, which is essential to achieve reliable single calibrant quantification with CAD.

Introduction

According to the World Health Organization, cancer is the second-leading cause of death globally, and was responsible for 8.8 million deaths in 2015. Worldwide, nearly 1 in 6 deaths is due to cancer.¹ There is considerable interest in the development of innovative drugs to support therapies (e.g., chemotherapy) or to treat patients individually with cutting-edge medication.

One example is paclitaxel (known under its tradename Taxol®), which belongs to the taxane family and can be either directly extracted from the bark of the Pacific yew (*Taxus brevifolia*) or be obtained by partial chemical synthesis from a precursor.

For any drug substance, unwanted impurities are a key concern during drug development and throughout a drug product's life cycle. Monitoring these impurities, which often are present in low abundance compared to the active pharmaceutical ingredient (API), is mandatory to ensure a safe and effective product. Even though some impurities may be closely related to the API, calibration standards are not likely to be available, particularly during early stages of development. Usually, (U)HPLC with UV/Vis detection is the preferred choice for analysis, but substances that lack a chromophore, or vary widely in their response factors, i.e. their extinction coefficients, are challenging to analyze. The issues with UV/Vis detection also apply to counter ion monitoring and especially to stability and degradation studies, which are necessary to support the product development. Consequently, it is often difficult to meet analytical requirements to demonstrate product quality. The uncertainties in the quantification make it challenging to accurately classify the impurities according to the ICH guidelines with respect to reporting, identification, or qualification threshold, which are in place to prevent severe side effects of the final drug formulation.²

CAD can be used to overcome these quantitation challenges (e.g., response or detectability issues). As CAD response is independent of the chemical structure of nonvolatile analytes, it is an ideal chromatographic approach when individual calibrants are unavailable.³ Under isocratic conditions, the calibration curve obtained with CAD using an available standard, e.g. reference standard of an API, can be used to quantify all nonvolatile analytes in a sample, within a certain range of confidence. As CAD response is affected by mobile phase composition, it is necessary to ensure that the universal response is applicable in gradient elution. This can readily be achieved through a compensation gradient delivered by the Thermo Scientific™ Vanquish™ Flex Dual Pump.⁴

In this application note, a Thermo Scientific™ Accucore™ Pentafluorophenyl (PFP) column was used to separate paclitaxel from its related compounds and other

impurities. Calibration using standards of paclitaxel and related compounds was used to estimate the quantities of unknown impurities present in the paclitaxel product. A thermal degradation study was also performed with the degradation products being analyzed and subsequently quantified using UHPLC-UV-CAD.

Experimental

Recommended lab consumables and equipment

- Accucore Pentafluorophenyl (PFP) column, 2.6 µm, 2.1 × 150 mm, L43 (P/N 17426-152130)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (P/N 50136171)
- Thermo Scientific™ Digital Heating Shaking Drybath (P/N 88880028)

Sample preparation

Calibration

The API (paclitaxel) and the related impurities C (Impurity C) and A (cephalomannine) were bought from the European (Strasbourg, France) and United States Pharmacopeia (Rockville, MD, United States), respectively. Baccatin III (purity 97%) was obtained from Sigma-Aldrich® (Schnellendorf, Germany) and was only used for peak identification. All calibration standards were accurately weighed and transferred to volumetric flasks and were brought to volume with methanol to achieve a final concentration of 0.1 mg/mL. A 10 µg/mL stock solution containing all three substances was prepared by transferring each of them into a volumetric flask and bringing to volume with methanol. Accordingly, the 10, 5, 1, and 0.5 µg/mL calibration standards were produced by a dilution series with methanol and each was analyzed in three consecutive runs with blank injections in between the different concentrations. Due to the limited stability in solution, standards were prepared directly prior to analysis.

Forced degradation

For the thermal degradation study, a 100 µL volume of a 1 mg/mL paclitaxel solution was diluted with 350 µL methanol and 50 µL dimethyl sulfoxide (DMSO) in a 1.5 mL Eppendorf tube. The solution was exposed to 65 °C for 2 hours using a Digital Heating Shaking Drybath. The degraded sample was then analyzed immediately without further sample preparation.

Instrumentation

The separation was achieved using an Accucore PFP column, which is well suited for the separation of aromatic compounds. Chromatographic conditions are summarized in Table 1. Detection was performed using the Thermo Scientific™ Vanquish™ Flex Variable Wavelength Detector followed by the Thermo Scientific™ Vanquish™ Flex Charged Aerosol Detector.

The Vanquish Flex Inverse Gradient LC system consisted of:

- System Base Vanquish Flex (P/N VF-S01-A-02)
- Dual Pump F (P/N VF-P32-A-01)
- Split Sampler FT (P/N VF-A10-A-02) with a 25 µL sample loop
- Column Compartment H (P/N VH-C10-A-02)
- Charged Aerosol Detector F (P/N VF-D20-A)
- Variable Wavelength Detector F (P/N VF-D40-A)
- Vanquish Inverse Gradient Kit (P/N 6036.2010)

Fluidic scheme

Figure 1 illustrates the Vanquish Inverse Gradient system setup. The right part of the Dual Gradient Pump delivers the eluent flow to the column (analytical gradient), and the left pump forms the second gradient (compensation gradient) directly to the T-piece immediately before the CAD.

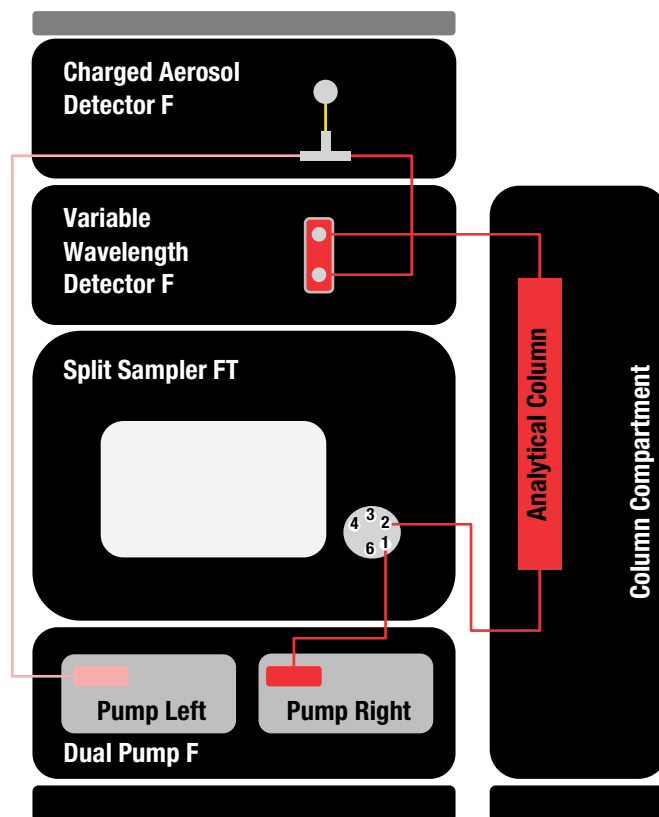


Figure 1. Fluidic scheme of the Vanquish Inverse Gradient system including a Charged Aerosol Detector F and Variable Wavelength Detector F.

Separation conditions

Table 1. Chromatographic conditions.

UHPLC Experimental Conditions

| | |
|------------------------|---|
| Column: | Accucore PFP 2.1 × 150 mm, 2.6 µm |
| Mobile phase: | A: Water, Ultra-pure (18.2 MΩ·cm at 25 °C) B: LC-MS grade acetonitrile |
| Analytical gradient: | 23–60 %B in 25 minutes; 0.3 mL/min |
| Compensation gradient: | 23–60 %A in 25 minutes, 0.3 mL/min |
| Temperature: | 35 °C Forced air; Active pre-heater 35 °C |
| Injection volume: | 1 µL |
| UV detection: | 227 nm, 5 Hz, Response time 1 s |
| CAD: | Evaporation Temp. 50 °C, 5 Hz, Filter 3.6 |

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.8 was used for data acquisition and evaluation.

Results and discussion

Global calibration

As mentioned above, the uniform response of the charged aerosol detector is routinely achievable under isocratic nebulization conditions. The nebulization is affected by solvent composition, so a change in organic solvent content during a gradient will lead to a change in detector response.⁴ To compensate for this effect, an inverse gradient is applied post column using the Vanquish Flex Dual Pump to provide a uniform mobile phase composition. The inverse gradient bypasses the column and mirrors the analytical gradient composition to achieve isocratic flow to the nebulizer. The difference in response in UV and CAD using the Vanquish Inverse Gradient LC system is shown in Figure 2 (right). This shows that the UV response for 10 µg/mL of three different analytes differed by as much as 63%, while that of CAD differed by only 2%. This once again illustrates

that the CAD response is independent of the chemical structure and response variation due to solvent gradients is minimal when using the Vanquish Flex Dual Pump to perform inverse gradient compensation.

The CAD calibration is linear for all three compounds in the concentration range from 0.5 to 10 ppm (Figure 2, left). The uniform response for the API and related components is demonstrated by the high similarity of the slopes of the calibration curves, and therefore the workflow is applicable to the measurement of related impurities. In addition, the slopes of the calibration curves show a relative deviation of average response of just 2%.

Analysis of API degradation products

Effect of Inverse Gradient compensation

The stressed API sample was analyzed using the Vanquish Inverse Gradient LC system with the Vanquish Flex Dual Pump and compared to the same system configuration without gradient compensation (Figure 3). Both setups can detect the same number of peaks, but a significant difference in peak response is noticeable.

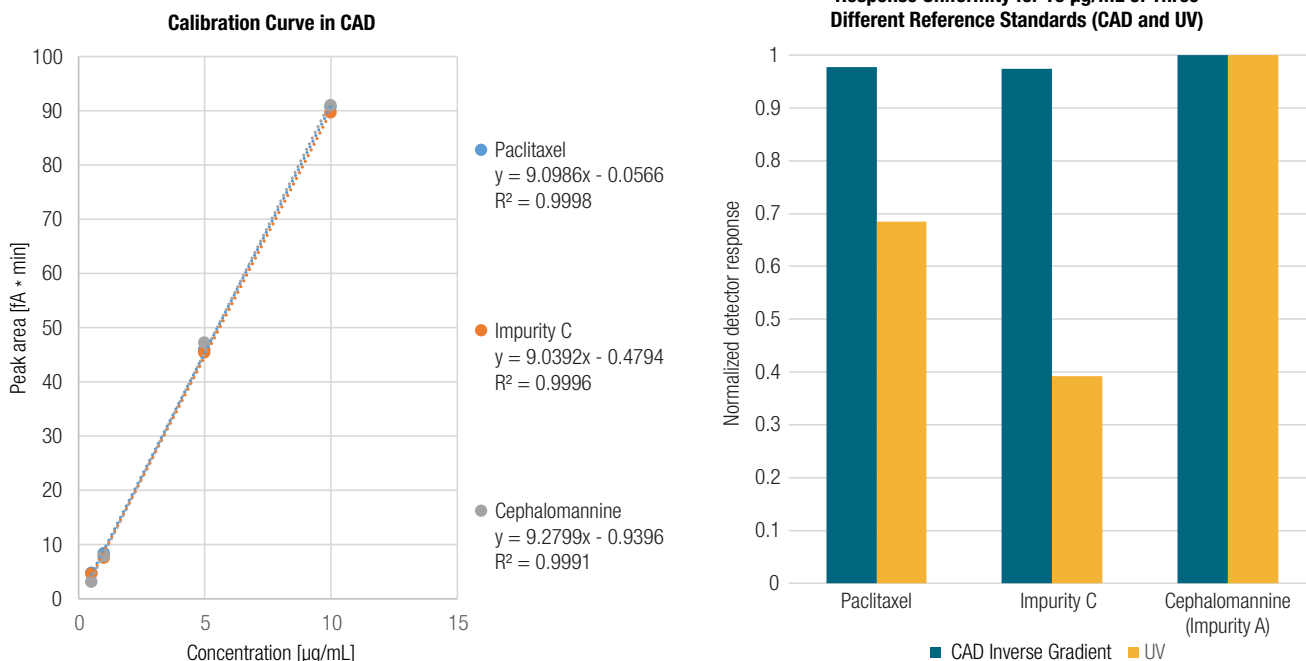


Figure 2. Uniform response was obtained using the Vanquish Inverse Gradient LC system with CAD as shown by the similar calibration curves obtained for the API and its related compounds (left side). By comparison, UV response was highly variable among analytes (right side). Response factors for both detectors were normalized to cephalomannine.

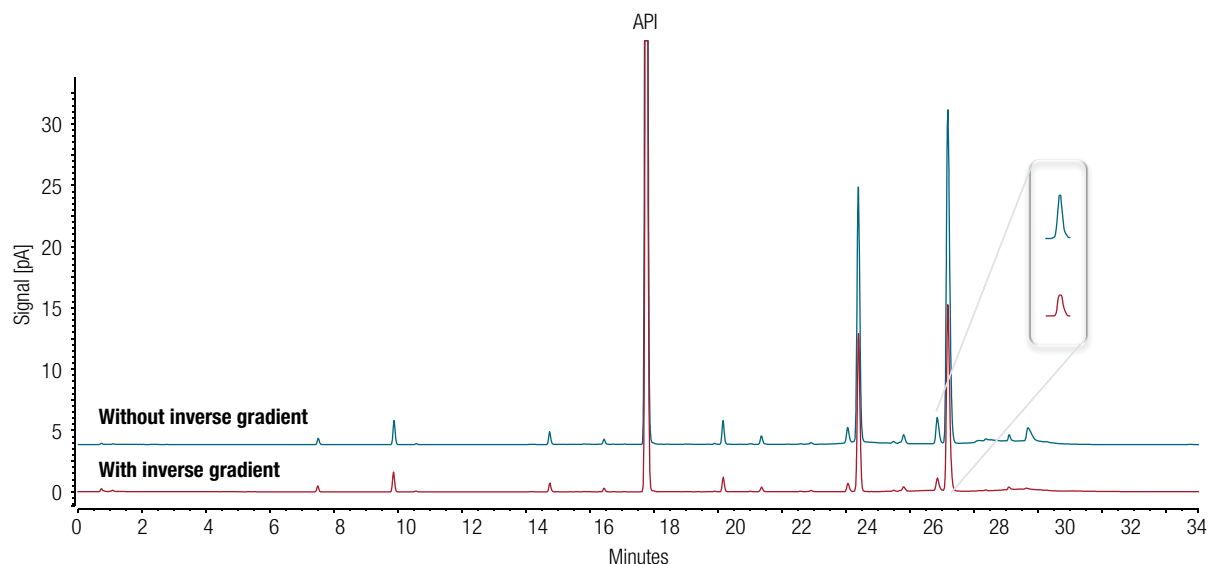


Figure 3. Comparison of CAD response with (red trace) and without (blue trace) applying inverse gradient compensation using the Vanquish Flex Vanquish Inverse Gradient LC system. Without inverse gradient, the quantity of analytes eluting before the API are underestimated while those eluting after the API are overestimated.

For the stressed sample shown in Figure 3, the combined peak areas for all impurities were found with CAD to be 63.9% of the API peak area when not using inverse gradient compensation (blue trace) and 53.8% of the API when using inverse gradient compensation (red trace). This large (>10%) difference in determined impurity content is attributed to the influence of solvent composition on CAD response. As expected in reversed-phase gradients, response factors for later eluting peaks are higher when not using inverse gradient compensation (see Figure 3 inset). These data highlight the capabilities of the Vanquish Inverse Gradient LC system to achieve uniform response with CAD and thus to minimize quantitation errors.

Comparison between UV and CAD

The combination of charged aerosol and ultraviolet absorbance detection can provide a more holistic approach when analyzing complex samples. As shown in Figure 4, some peaks lack UV activity and therefore

cannot be measured using single wavelength UV at 227 nm. One of the degradation products eluting at 6.6 minutes is only visible using UV detection since it is too volatile to be detected by CAD. The two main impurities (24.3 and 27.1 min) are only detectable in CAD.

Comprehensive analysis of degradation products of new drugs is feasible using the multi-detector approach with UV and CAD. An example degradation product (peak at minute 9.8) with similar retention behavior as the precursor baccatin III can now be quantified using global calibration (Figure 2). Here, the example compound has a relative area with respect to the API of 1.1%. The relative standard deviation (n=5) of the peak area precision is 0.25% for the peak eluting at minute 9.8 and 0.76% for the API. The Vanquish Inverse Gradient LC system, utilizing both CAD and UV, is an extremely powerful tool because of the complementary nature of the two detectors and is well suited for stability or degradation studies.

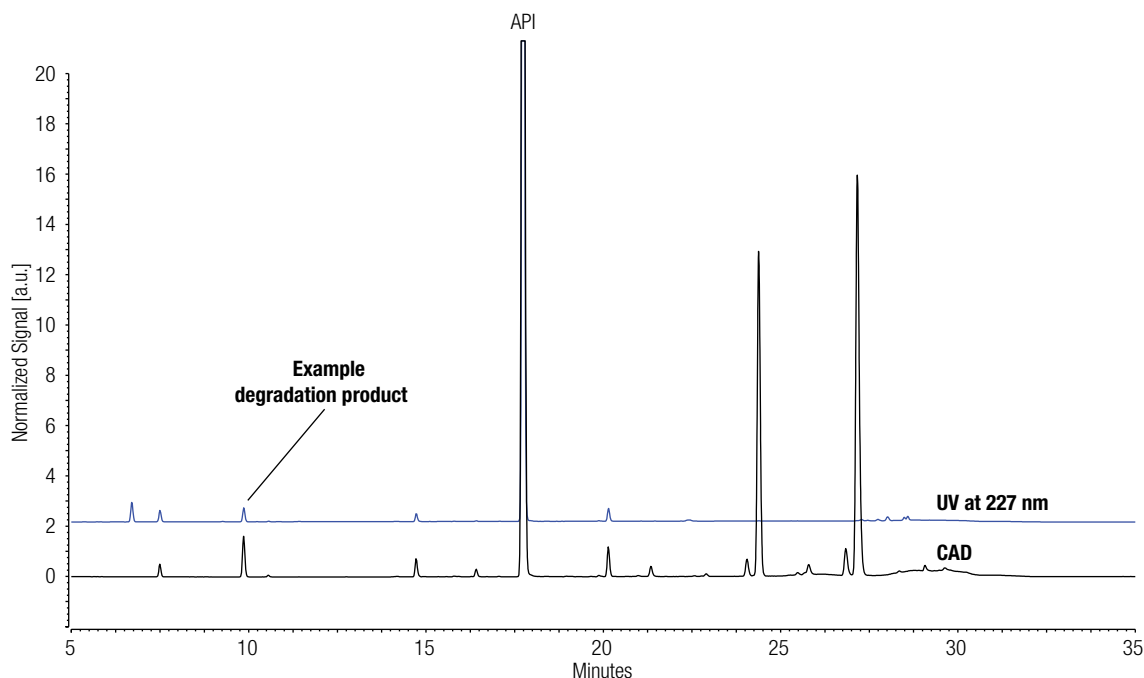


Figure 4. Comprehensive analysis of degradation products of paclitaxel using UV (blue) and CAD (black). UV signal normalized to the API.

Conclusion

Uniform response for degradation analysis with charged aerosol detection is easily achievable with a Vanquish UHPLC system and a post-analytical column inverse gradient. The combined analytical and inverse gradient flow yields a constant eluent composition in the CAD nebulizer, resulting in uniform response even during gradient runs. The proof of concept is presented here using the anti-cancer drug paclitaxel and two related impurities, which showed a variation in calibration slope of only 2%. Furthermore, the Inverse Gradient Workflow was successfully applied to impurity analysis of a paclitaxel standard resulting in a measurably more uniform analyte response throughout the gradient. Using the Vanquish Inverse Gradient LC system, the measured

amounts of analytes were corrected by more than 10%, thus giving a more accurate and unbiased determination of critical impurities. When combined with UV detection, the resulting comprehensive profiling of a new drug candidate, its impurities, degradants, and related substances is straightforward, making this multi-detector setup a powerful tool for analytical laboratories.

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