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Quantitation of tenofovir and impurities in multi-component drug products by ternary gradient reversed-phase chromatography with charged aerosol detection

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Keywords

Tenofovir, emtricitabine, charged aerosol detector, uniform response, inverse gradient compensation, impurity profiling, ternary gradient, single-calibrant quantitation

Goal

Demonstrate the implications of salt formation on uniform response when using charged aerosol detection.

Application benefits

- Improves single-calibrant quantitation of charged analytes by taking into consideration their salt formation with mobile phase additives
- Illustrates the ability to use inverse gradient compensation to normalize detector response when using a ternary (three-eluent) gradient

Introduction

The antiretroviral drug combination of emtricitabine and tenofovir disoproxil (TD) is used to treat HIV patients. Impurity profiling by reversed-phase chromatography is difficult because both early-eluting polar as well as late-eluting hydrophobic impurities are present (see Figure 1 for chemical structures). A previously developed method used a ternary gradient with UV detection to profile the impurities within 10 minutes.[1] Unfortunately, UV detector response depends upon the nature of the chromophore present. The two active pharmaceutical ingredients (APIs) measured had very different extinction coefficients, so quantitation by UV detection required individual standards for the APIs and all impurities[2]—a major drawback for many methods where individual reference standards are not available.

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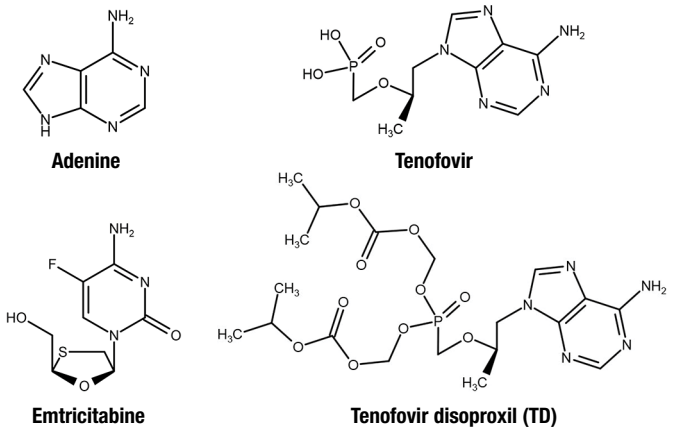

Figure 1. Structures of components in the test mixture

Charged aerosol detection shows uniform response for all nonvolatile compounds, independent of the chemical structure. It therefore allows quantitation of all nonvolatile components in the sample with a **single** calibrant. Although the response of the charged aerosol detector (CAD) is independent of the nature of the molecule, it is dependent on mobile phase solvent composition. Specifically, response during a gradient will increase as the amount of organic solvent increases. In order to address this issue, a compensation gradient is applied post column so that the CAD always experiences the same solvent composition and the detector response is uniform. This approach has been extensively applied to binary gradients only. However, due to the analytical limitations of the binary gradient, a ternary gradient is required to rapidly separate the critical peak pair adenine and tenofovir with good resolution.[1]

In this work, the ability to use an inverse ternary gradient and its application to the analysis of a mixture of antiviral drugs is shown for the first time. With the ternary gradient, CAD quantitative accuracy with and without inverse gradient is compared. Comparison of the quantitative accuracy of the CAD and a UV detector is also evaluated. Finally, a simple strategy to account for salt formation between charged analytes and mobile phase additives (acetate in this example) on detector response is presented.

Experimental

Chemicals and water system

- Acetonitrile, Fisher Scientific™ Optima™ LC/MS grade (P/N A955)
- Methanol, Fisher Scientific™ Optima™ LC/MS grade (P/N A456)
- Acetic acid, Fisher Scientific™ Optima™ LC/MS grade (P/N A113)
- Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (P/N 50136171)

Instrumentation

Thermo Scientific™ Vanquish™ Flex Duo UHPLC system for Inverse Gradient consisting of:

- System Base (P/N VF-S01-A-02)
- Dual Pump F (P/N VF-P32-A-01)
- Split Sampler FT (P/N VF-A10-A-02)
- Column Compartment H (P/N VH-C10-A-02)
- Thermo Scientific™ Dionex™ Corona™ Veo™ / Thermo Scientific™ Vanquish™ Charged Aerosol Detector F (P/N VF-D20-A)
- Variable Wavelength Detector F (P/N VF-D40-A)
- Capillaries as shown in Figure 2 or Thermo Scientific™ Vanquish™ Duo for Inverse Gradient Capillary Kit (P/N 6036.2010)

Sample preparation

USP standards were used for tenofovir, emtricitabine, and tenofovir disoproxil fumarate. Samples of 1.0 mg/mL were prepared in water. Adenine was prepared at 0.1 mg/mL in 0.1% acetic acid for solubility reasons. Tenofovir disoproxil fumarate calibration standards were prepared at 2000, 1000, 500, 200, 100, 50, 40, 30, 20, 10, 7.5, and 5 µg/mL in water. All other analytes were prepared at concentrations of 50, 40, 30, 20, 10, 7.5, and 5 µg/mL in water. Samples were measured in quintuplet. TD is formulated as the fumarate salt, but fumarate separates from TD on the column. Therefore, 1 mg of salt contains 0.82 mg of TD. This is taken into account when evaluating the calibration curves.

Mobile phase preparation

Eluent A was prepared by adding 1 mL acetic acid to 1000 mL water and the resulting pH was 3.5. Mass spectrometric-grade methanol and acetonitrile were used as eluents B and C, respectively. Organic solvents were refreshed weekly to reduce background noise.

Chromatographic conditions

Table 1. Chromatographic conditions for the impurity analysis of TD fumarate

Column:	Thermo Scientific™ Accucore™ aQ, 2.6 µm, 2.1 × 100 mm (P/N 17326-102130)
Mobile Phase:	A – water with 0.1% acetic acid, pH 3.5 B – methanol C – acetonitrile
Gradient:	0–4 min: 0–70% B, 0–15% C 4–4.5 min: 70% B, 15% C 4.5–5 min 70–25% B, 15–70% C 5–6 min: 25% B, 70% C 6–6.1 min: 25–0% B, 70–0% C 6.1–15 min: 0% B, 0% C
Inverse Gradient:	0–4 min: 70–0% B, 25–10% C 4–4.5 min: 0% B, 10% C 4.5–4.591 min 0–8% B, 10–0% C 4.591–6 min: 0% B, 0% C 6–6.1 min: 0–70% B, 0–25% C 6.1–15 min: 70% B, 25% C
Flow Rate:	0.6 mL/min
Column Temp.:	40 °C still air mode 40 °C active preheater
Autosampler Temp.:	4 °C
Injection Volume:	1 µL; 5 µL for flow injection analysis
CAD Settings:	Evaporator Temp.: 35 °C, Filter: 3.6 s, Data Collection Rate: 20 Hz, Power Function Value: 1.00
UV Detector Settings:	Wavelength: 260 nm, Data Collection Rate: 10 Hz, Response Time: 0.50 s
Data Processing:	Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), 7.2.8

Flow paths

The flow paths for the analytical and inverse gradients are shown in Figure 2. Two columns were used. The column in the flow path of the second, compensatory, gradient removes impurities in the solvent that may be detectable in the CAD as baseline variations. Even MS-grade solvents can contain impurities that are MS-invisible but CAD-visible.

Gradients

The relationship between analyte quantity and CAD response changes during gradient elution because of the influence of eluent solvent composition on the nebulization process. A dual pump approach was therefore adopted to normalize response between analytes. One pump produced the analytical gradient that flowed through the autosampler and analytical column, and a second pump produced a compensation gradient to ensure that the CAD experienced a constant solvent composition. The flow path is shown in Figure 2. When using gradient compensation, it is important to incorporate a delay time for the inverse gradient to account for any differences in volume between the analytical and compensation flow paths (in this case due to the extra dwell volume of the autosampler). This delay time is easily programmed into the gradient method using the fluidics wizard in Chromeleon CDS, which is described in detail in Reference 3. With binary gradients, it is relatively straightforward to match the analytical gradient with an exact inverse compensation gradient. In the binary gradient example shown in Figure 3, the CAD always receives 50% of eluent B. For a ternary gradient, it is not always possible for the compensation gradient to match the analytical gradient through the entire run. The ternary gradient used in this application is shown in Figure 4. The first 4.5 min of the analytical gradient is used to separate all analytes, and during this time, the solvent composition was effectively matched with an inverse gradient. After 4.5 min, the analytical gradient is used to wash the column. During this wash step, no analytes elute and the analytical gradient is not fully compensated.

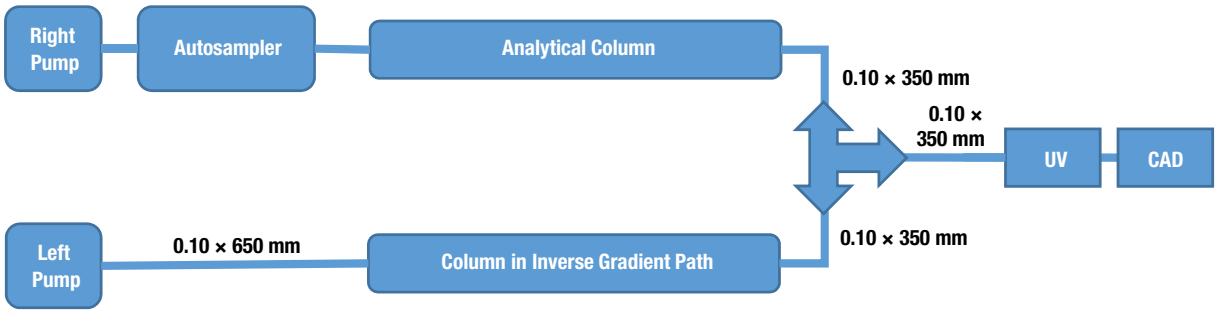

Figure 2. Flow path for the inverse gradient

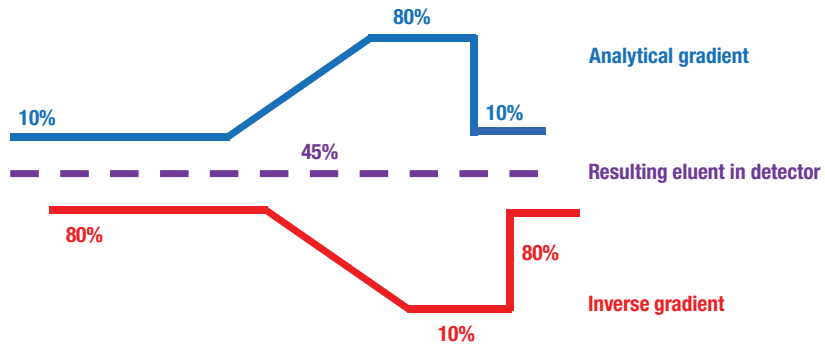

Figure 3. Example of the inverse gradient for eluent B of a conventional two-eluent system. The timing of the inverse gradient is delayed because the flow path is shorter. One reason the inverse gradient flow path is shorter is because the inverse gradient does not pass through the autosampler. The wizard sets a constant eluent composition depending on the analytical gradient. Optimal conditions for uniform response are a constant eluent composition throughout the gradient method, but not necessarily a 50:50 composition.

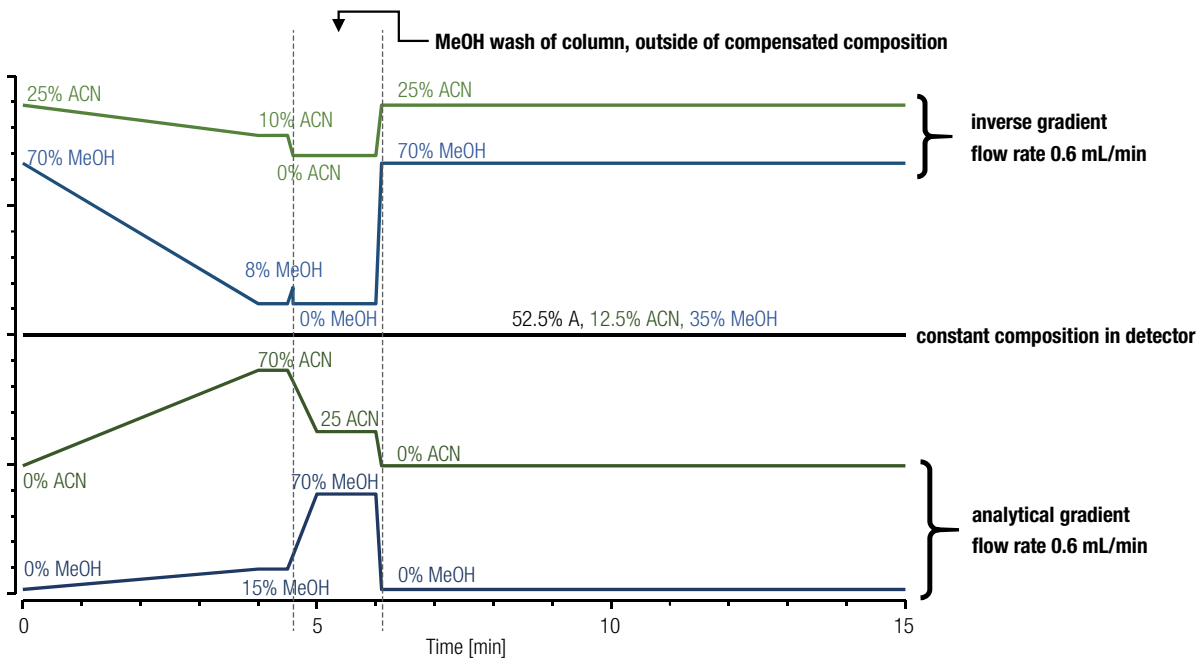

Figure 4. Ternary gradient as implemented with analytical (bottom) and inverse (top) gradients. The resulting composition in the detector is 52.5% A, 12.5% acetonitrile, and 35% methanol.

Results and discussion

Among common HPLC detectors, the CAD provides the most uniform response across nonvolatile analytes while also detecting many semivolatiles. Because of the CAD's response uniformity where the measured signal magnitude is similar for numerous nonvolatile analytes and impurities at the same concentration, a single calibrant (one of the APIs in this example) can be used for the quantitation of impurities. This is a major advantage over other analytical approaches that require the availability of impurity standards.

Advantages of inverse gradient

Figure 5 shows the calibration curves without (A) and with inverse gradient (B). The inverse gradient serves to normalize the peak height and area relative to the analytical gradient and to reduce baseline drift. More information on the inverse gradient is provided in a recent technical note on the topic of CAD response uniformity.[3]

The compensating effect of the inverse gradient is shown in the chromatograms in Figure 6. Without compensation, analyte response increases as the organic content of the mobile phase increases, so that the response of the late-eluting TD is much greater than that of the early elutors. When gradient compensation is applied all peaks areas match and, as seen in Figure 5, all response curves match. Table 2 shows that use of the inverse gradient brought the peak areas of all four analytes to about 8% RSD of each other at 30 ng, almost a ten-fold improvement on the 74% RSD of the peak areas without the inverse gradient. As described below for analytes that elute as charged solutes, further improvement in response uniformity and therefore accuracy of single-calibrant quantitation can be achieved by correcting for salt formation.

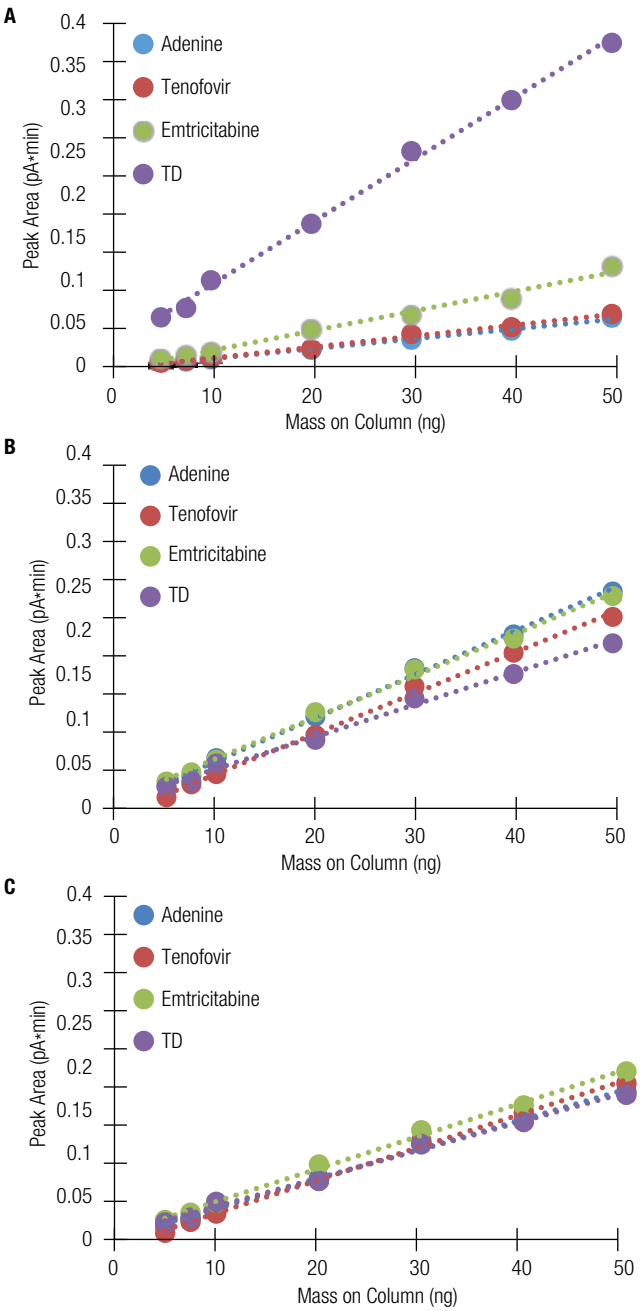

Figure 5. Calibration curves for TD and impurities (A) without inverse gradient compensation, (B) with inverse gradient compensation, and (C) with inverse gradient compensation and salt correction assuming that all of the analytes exist as singly charged cations (+1) in the eluent

Table 2. Comparison of single calibrant data for 30 ng before and after salt correction. The salt correction calculation assumed one acetate per molecule.

Analyte	UV (ng)	No salt correction No inverse gradient (ng)	Salt correction No inverse gradient (ng)	No salt correction Inverse gradient (ng)	Salt correction Inverse gradient (ng)
Adenine	149	1.2	0.2	41	32
Tenofovir	87	2.1	1.7	36	33
Emtricitabine	44	5.2	4.3	41	37
% RSD	57%	74%	99%	8.1%	7.7%

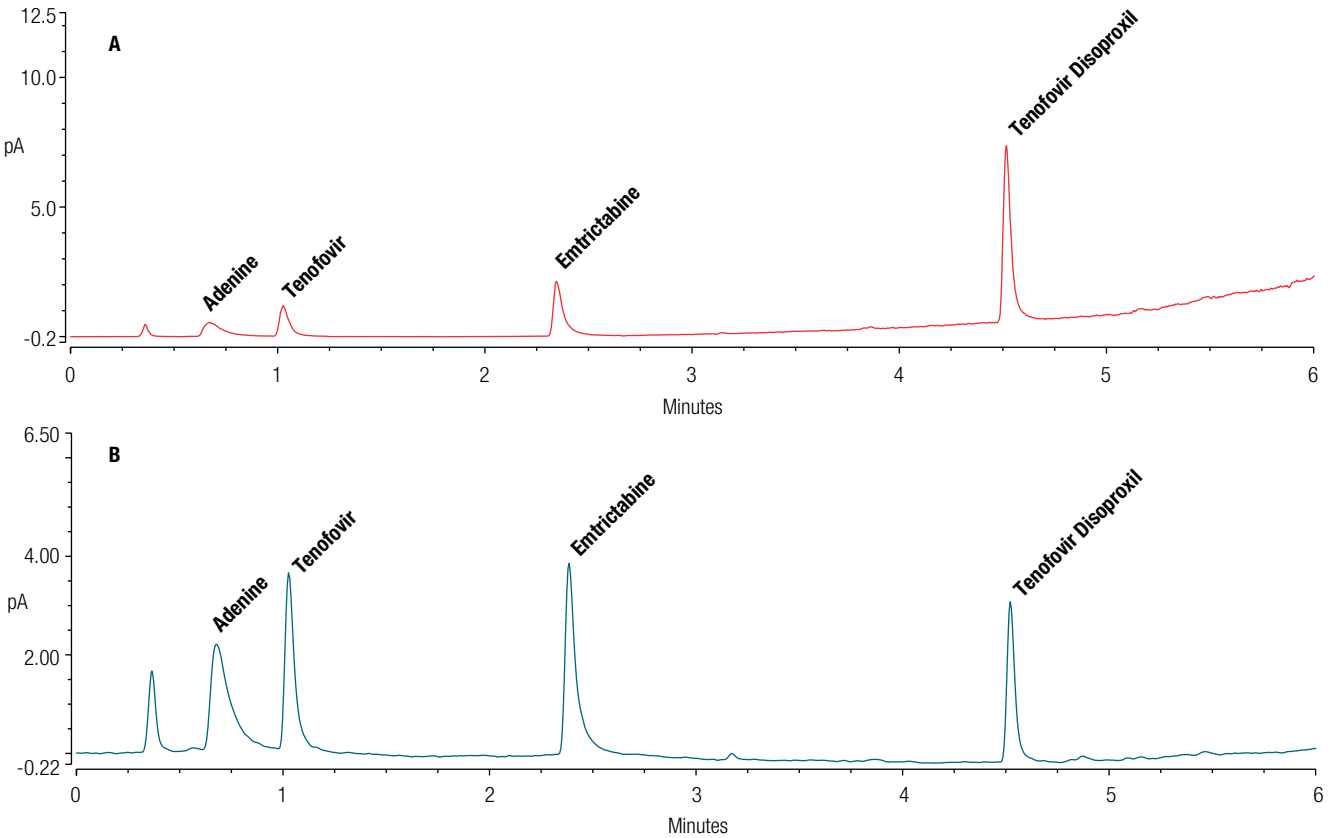

Figure 6. Comparison of chromatograms for 20 ng on column of each substance either without (A) or with (B) inverse gradient compensation

An analysis of 30 ng of all substances quantified using the TD calibration curve is shown in Figure 7. Concentrations of adenine, tenofovir, and emtricitabine were evaluated as if these three substances were unknown impurities for which calibration curves could not be generated. Quantitative values obtained by UV, CAD without gradient compensation, and CAD with gradient compensation are compared. The latter results were also corrected for salt formation as detailed below. The quantitation of API and impurities using TD as single calibrant is by far the most accurate when using CAD with inverse gradient compensation. With this approach, variation (RSD) of amount among analytes was only 7.7%. By comparison, values obtained by single-calibrant quantitation differed by as much as 5-fold (57% RSD) when using UV and up to 25-fold (99% RSD) when using CAD without inverse gradient compensation. As noted above, the 7.7% RSD among analytes was obtained with both gradient compensation and correction for salt formation. Additional factors, unrelated to detection, that may contribute to this variation may include analyte purity, stability, and hygroscopicity of powder.

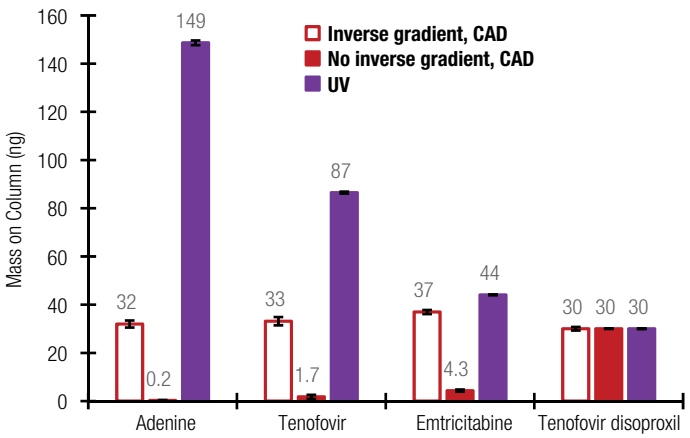

Figure 7. Quantitation results for 30 ng of each analyte using TD as a single calibrant by CAD both with and without the inverse gradient, and by UV detection. The CAD datasets include corrections for acetate salt formation.

Correction for salt formation

In the particles resulting from the CAD evaporation process, analytes that are either positively or negatively charged in solution will associate with a counterion to form salts. As these salts have higher masses than the analytes themselves, response will increase. Mobile phase counterions are particularly likely to influence response and increase analyte mass because of their high concentration. An extensive technical note on CAD uniform response provides additional information on various salt formation strategies and CAD response.[3]

All analytes in the tested sample mixture are likely to carry a +1 charge at pH 3.5 because the isoelectric point of each of the analytes is above 3.5. One nitrogen heteroatom of each molecule is singly positively charged at this pH. The only acidic functional group, the phosphonic acid of tenofovir, carries no charge at pH 3.5. They all therefore almost certainly are associated with an acetate counterion from the excess acetate in the mobile phase.[4] The following simple response correction accounts for the increased mass of the analyte due to salt formation:

$$\text{Corrected Response} = \text{Response} * \frac{M_w\,(\text{analyte})}{M_w\,(\text{analyte + counterion})}$$

Using the assumption of one acetate per molecule, the estimated concentrations for 30 ng of adenine, tenofovir, and emtricitabine using the dataset collected with the inverse gradient are summarized in Table 2. Calibration curves for these two cases are shown in (B) and (C) of Figure 5. When not corrected for salt formation, masses were overestimated by up to 33%.

If the ionization state is unknown, as in the case of impurities or other unknown substances in a sample, the effect of salt formation can be minimized by choosing low molecular weight mobile phase additives. For example, formic acid adds only 45 Da to the molecular mass of a substance and is preferable to trifluoroacetic acid (TFA) (+113 Da), acetic acid (+59 Da), or heptafluorobutyric acid (HFBA) (+213 Da). Therefore, although a higher mass salt generally results in improved limits of detection, a lower mass salt improves quantification accuracy of unknowns.

The low pH mobile phases, such as 0.1% formic acid, that are common in reversed-phase chromatography ensure that most acidic analytes are neutral while most basic analytes are protonated. This pH choice controls protonation state distribution, thereby reducing peak tailing and retention time instability. Under these conditions, basic analytes will gain mass due to salt formation and acidic analytes will be largely unaffected.

Flow injection analysis to determine salt correction strategy

Flow injection analysis, where the separation column is replaced with a pressure coil, permits a rapid study of analytes whose individual standards are available. This can be used to examine the influence of mobile phase pH and the additive type (molecular weight) on analyte response. Use of this technique allowed for a more rigorous evaluation of salt formation with mobile phase counterions. Additional examples of salt formation analysis by flow injection analysis are discussed in a technical note.[3]

A 500 ng sample of each compound was analyzed with a mobile phase of 52.5% A, 35% B, and 12.5% C, when A was either water or 0.1% acetic acid, B was acetonitrile, and C was methanol. Theoretically, as analytes are charged at pH 3.5 and acetate is present in excess in the nebulized droplet, salt formation will increase analyte mass and detector response.[4] The results confirm that introduction of acetate adds mass to all four substances (Figure 8). Correcting peak areas by assuming the detector is responding to one acetate per molecule yields a very low 6.2% relative standard deviation in peak area for these four different analytes (Table 3). These results support the conclusion that if information about the expected protonation of the impurity is known in advance assumptions about salt formation can be integrated into the calibration curve. When individual standards are not available, unknowns can be studied chromatographically by examining the effect of different molecular weight additives (e.g., formic acid vs. TFA at the same pH) on response.

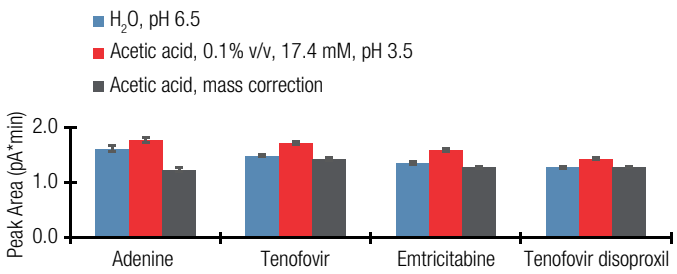

Figure 8. Results of the flow injection experiment that quantified the effect of pH on response. A restriction capillary and injections of 500 ng were used. Adenine was prepared in 0.1% acetic acid instead of water for solubility reasons.

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Table 3. Peak areas from flow injection

	Water, pH 6.5 (pA*min)	Acetic acid, 0.1% v/v, 17.4 mM, pH 3.5 (pA*min)	Acetic acid, mass corrected for one acetate per molecule (pA*min)
Adenine	1.62	1.77	1.23
Tenofovir	1.48	1.72	1.42
Emtricitabine	1.36	1.59	1.28
TD	1.28	1.43	1.29
% RSD	**0.10**	**0.09**	**0.06**

Conclusions

- Inverse gradient compensation enables impurity quantitation of unknown substances by compensating for differences in nebulizer efficiency during the gradient.
- A ternary gradient offers greater chromatographic flexibility and can be used with an inverse gradient produced by a dual gradient pump.
- Analytes that are charged in solution may form salts with mobile phase additives. The influence of mobile phase additive on CAD response can be minimized by using a low molar mass additive (formic acid better than acetic acid) and maximized with larger molar mass additives like TFA and HFBA. The effect is more pronounced for lower molar mass analytes, especially volatile analytes
- A single calibrant can be used to quantify levels of other substances in a sample, such as impurities for which no standard is available. For gradient methods, inverse gradient compensation is recommended. For ionizable analytes, corrections for possible salt formation can provide the most accurate results.

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