thermo scientific

Quantitation of APIs and impurities in multi-component drugs by ternary gradient reversed phase chromatography with charged aerosol detection

Katherine Lovejoy, Mauro de Pra and Frank Steiner
Thermo Fisher Scientific, Dornierstr. 4, Germering, Germany 80798

ABSTRACT

The antiretroviral drug combination of emtricitabine and tenofovir disoproxil is used to treat HIV patients. The impurity profiling by reversed phase is difficult because the mixture contains early-eluting polar and hydrophobic impurities. A previously developed ternary gradient profiled the impurities within 10 minutes [1]. The method relied on UV detection. With UV detection, quantitation of the two active pharmaceutical ingredients (API) can only be achieved by calibration with two standards at different levels, due to the extinction coefficient difference [2]. The Charged Aerosol Detector (CAD) is regarded as universal detector, thanks to very similar response to semi- and non-volatile molecules. This means that, in principle, quantitation of different entities can be achieved by drawing just one calibration curve obtained from one of the components to be analyzed. However, even if independent from the nature of the molecule, response depends on the solvent that needs to be nebulized. Consequently, response during a gradient will vary based on the mobile phase composition. An established practice in CAD with gradient chromatography is to apply an inverse gradient that will merge with the gradient delivered through the column at a point between the column outlet and the detector nebulizer. In this way, the composition of the effluent entering the nebulizer of the detector will be constant, and the detector response will remain stable. This approach has been extensively applied to binary gradients. In this work, the mixture of the antiretroviral drug is used to demonstrate the inverse ternary gradient in analysis of drugs based on multiple APIs. The quantitation accuracy and sensitivity of the CAD with and without inverse gradient are compared. Moreover, quantitation of the related impurities by CAD and UV detection is compared.

INTRODUCTION

The charged aerosol detector provides the most consistent response across all nonvolatile and some semivolatile analytes of all HPLC detection techniques. The detector is mass sensitive and can be added to the traditional HPLC-UV platform. Because CAD has a consistent relationship for most analytes between the quantity injected and the magnitude of response, we can use the response of a drug to quantify impurities of the drug, even though the impurities might have very different structures (Figure 1). The relationship between quantity and response is not the same for all solvent mixtures, so we use one pump to produce the gradient and a second pump to ensure that the CAD always sees the same solvent mixture. The flow path is shown in Figure 2. By using the second pump to provide a make-up flow, a gradient such as in Figure 3 is produced. In this example, the CAD always sees 50% of solvent A. For a ternary gradient, the highest percent concentration of all three solvents must sum to 100 or less

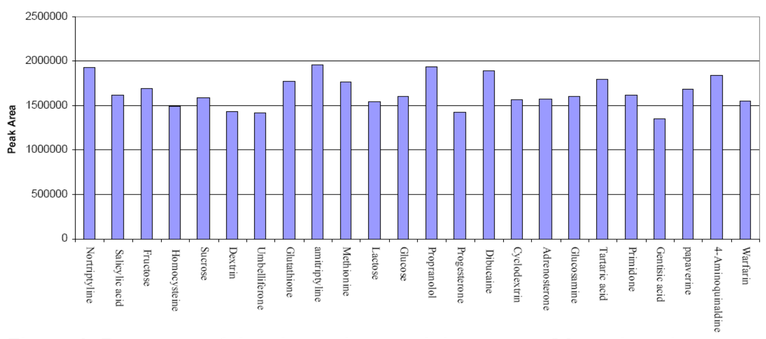


Figure 1. Response of the charged aerosol detector to 24 semi- and non-volatile analytes, analyzed by flow injection at 1.0 µg.

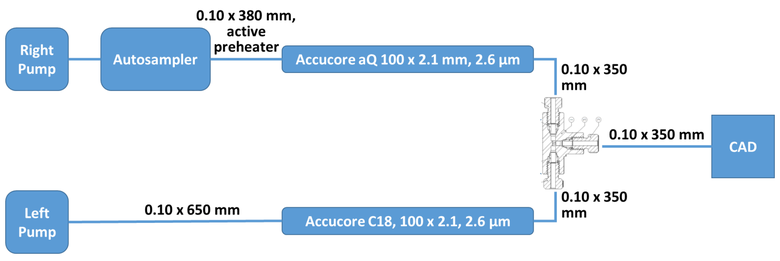


Figure 2. Flow path for the inverse gradient.

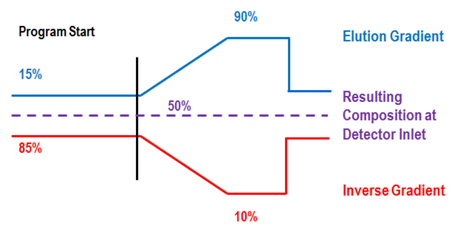
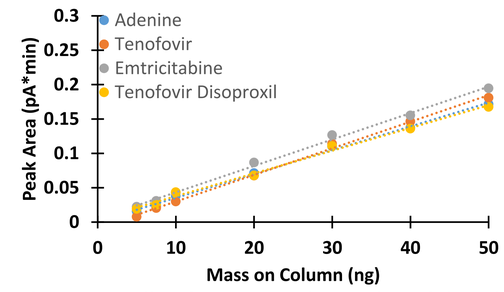


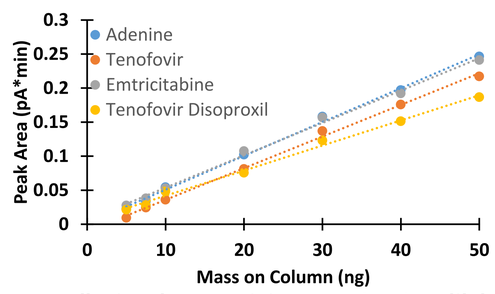
Figure 3. Example inverse gradient for one solvent of conventional two-solvent system.

MATERIALS AND METHODS

Table 1. Chromatographic Conditions

Column:	Thermo Scientific™ Accurcore™ aQ, 2.6 µm, 2.1 x 100 mm (P/N 17326-102130)
Mobile Phase:	A – water with 0.1% acetic acid 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
Flow Rate:	0.6 ml/min
Temperature:	40 °C still-air mode, active preheater at 40 °C, Vanquish TCC
Injection Volume:	1 µL. 5 µL for flow injection, Vanquish Flex Autosampler
CAD Detection:	Thermo Scientific™ Vanquish™ Flex CAD, Evaporator Temp.: 35 °C, Filter: 3.6, Data Collection Rate: 20 Hz, Power Function Value: 1.00
Data processing:	Thermo Scientific™ Dionex™ Chromeleon™ CDS 7.2 SR5





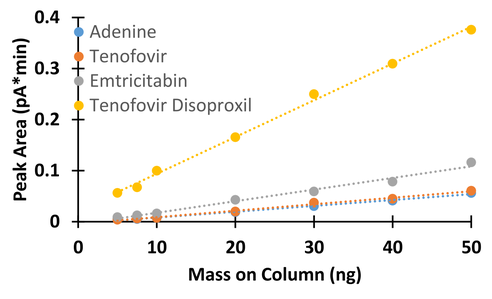


Figure 4. Calibration curves, from left to right, with inverse gradient and one acetate per molecule, with inverse gradient and no acetates, without inverse gradient.

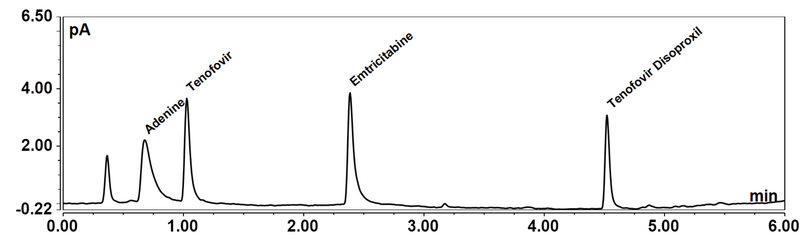


Figure 5. 20 ng on column with inverse gradient.

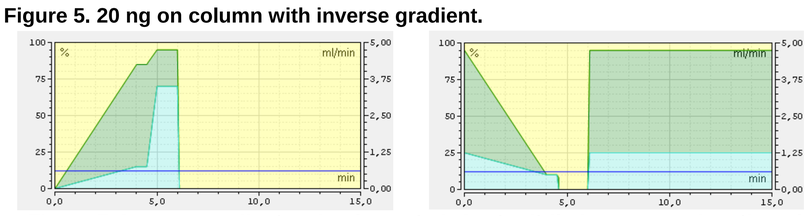


Figure 7. Inverse gradient as implemented. Acetonitrile is green and methanol is blue.

Two columns were used in the flow path (Figure 2) to ensure the gradient delay volume would be identical for the gradient and inverse gradient. Another option would be to calculate the gradient delay volume for both flow paths and add an appropriate delay to the start of the inverse gradient. USP standards were used for tenofovir, emtricitabine and tenofovir disoproxil fumarate and samples of 1 mg/ml were prepared in water. Adenine was prepared at 0.1 mg/mL in 0.1% acetic acid for solubility reasons. Tenofovir disoproxil calibration standards were prepared at 2000, 1000, 500, 200, 100, 50, 40, 30, 20, 10, 7.5, and 5 ng in water. All other analytes were prepared at concentrations of 50, 40, 30, 20, 10, 7.5 and 5 ng in water. Samples were measured in quintuplet.

RESULTS

Tenofovir disoproxil is supplied as the fumarate salt, but fumarate does not elute with tenofovir disoproxil. Therefore, 1 mg of salt will only contain 0.82 mg of tenofovir disoproxil, a fact that we considered when evaluating the calibration curves, but which also could be accommodated during sample preparation. The ternary gradient for tenofovir requires up to 95% A, up to 70% B and up to 70% C. This is impossible to program as an inverse gradient. Luckily, the last analyte to elute, tenofovir disoproxil, elutes before the inverse gradient fails to fully compensate. After it elutes, the column is only washed and the solvent at the detector is not important for quantification. Without the inverse gradient, the largest peak is tenofovir disoproxil, which elutes in the solvent with highest organic content (Figure 6). Mobile phase with lower viscosity, lower surface tension and increased volatility leads to higher mass transport efficiency and a higher signal for analytes eluting late in the gradient. With the inverse gradient, all peaks match (Figure 5). Use of the inverse gradient brought the peak areas of all four analytes to within 12 %RSD of each other at 30 ng, almost a ten-fold improvement on the 111 %RSD of the peak areas without the inverse gradient.

Reinjection Analysis

Concentrations of adenine, tenofovir and emtricitabine were evaluated based on a calibration curve produced for tenofovir disoproxil, as if the three substances were unknown impurities for which calibration curves could not be generated. Without the inverse gradient, the estimated concentration of 30 ng adenine was 1.2 ng (Table 2), based on the calibration curve of tenofovir disoproxil (Figure 4). The estimations for 30 ng were 1.7 ng for tenofovir and 4.8 ng for emtricitabine. With the inverse gradient and the calibration for tenofovir disoproxil (Figure 4), the estimated concentrations for 30 ng of adenine, tenofovir, and emtricitabine were 38.3, 34.9 and 40.1 ng, respectively. The fact that all impurity concentrations are overestimations would help the user estimate whether impurities are present at critical levels. Because all of the analytes are likely to carry a +1 charge at pH 3.5 in the solid particles that result after the evaporation tube, they almost certainly carry an acetate counterion from the excess acetate of the mobile phase. Using the assumption of one acetate per molecule, the estimated concentrations for 30 ng of adenine, tenofovir, and emtricitabine are 29.6, 32.5, and 36.2 ng.

Table 2. Inverse gradient fit and calibration with tenofovir disoproxil curve.

Analyte	R^2, inverse gradient curves	Reinjection, 30 ng, with inverse gradient and 1 acetate per molecule	Reinjection, 30 ng, with inverse gradient	Reinjection, 30 ng, without inverse gradient
Adenine	0.9971	29.6	38.3	1.2
Tenofovir	0.9975	32.5	34.9	1.7
Emtricitabine	0.9964	36.2	40.1	4.8
Tenofovir disoproxil	0.9942	32.0	31.8	31.6

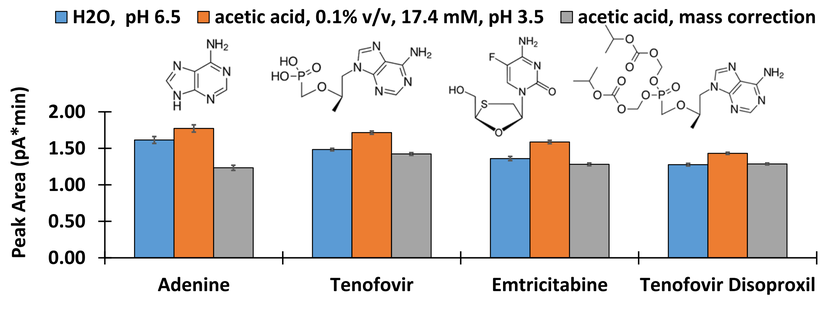


Figure 9. Results of the flow injection experiment that quantified the effect of pH on response. A restriction capillary and injections of 500 ng were used. Adenine was prepared in 0.1% acetic acid instead of water.

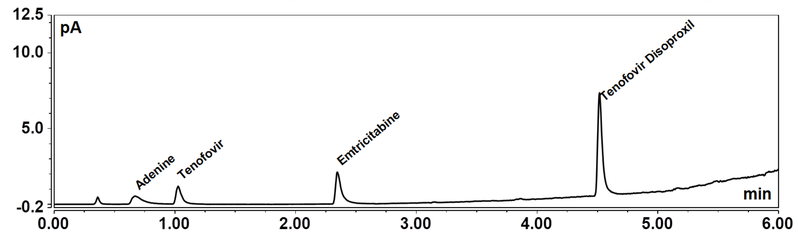


Figure 6. 20 ng on column without inverse gradient.

Figure 8. Ternary gradient for analysis of tenofovir with a quaternary pump.

Flow Injection Analysis

Differences in peak area even with the inverse gradient (Figure 4 and Table 2) may be due to factors unrelated to detection including analyte purity, stability and hygroscopicity of powder. In addition to gradient effects, ionic interactions between analytes and mobile phase counterions increases analyte mass and influences response. To study this we used flow injection analysis to evaluate ionic interactions with mobile phase counterions during droplet evaporation. Injections of 500 ng of each analyte were 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. The analytes are charged at pH 3.5 and acetate is present at large excess in the nebulized droplet. Consequently, salts should form and add to the mass of the analytes [3]. The results (Figure 9) confirm this, showing that introduction of acetate adds mass to all four substances. 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.

Table 3. Peak areas from flow injection.

	Water, pH 6.5	Acetic acid, 0.1% v/v, 17.4 mM, pH 3.5	Acetic acid, mass corrected for one acetate per molecule
Adenine	1.62	1.77	1.23
Tenofovir	1.48	1.72	1.42
Emtricitabine	1.36	1.59	1.28
Tenofovir Disoproxil	1.28	1.43	1.29

CONCLUSIONS

- The inverse gradient enables impurity quantification of unknown substances by compensating for differences in nebulizer efficiency during the gradient.
- A ternary gradient can be used with an inverse gradient produced by a dual gradient pump, provided that the maximum %A, %B and %C do not sum to more than 200.
- If salts, acids, or bases are present in any solvent and the analytes are charged, salts will form in the solid particles detected by the CAD. In the case of tenofovir, this leads to a slight overestimation of the “impurities,” which is more desirable than an underestimation.
- 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.

REFERENCES

[1] Fabel, S.Ternary Gradient for Tenofovir Disoproxil Fumarate Impurity Profiling, Thermo Fisher Scientific Application Note 1129
[2] Iavanya, B. *et al.* Method Development and Validation of Combined Tablet Dosage Form of Emtricitabine and Tenofovir Disproxil Fumerate by Ultraviolet Spectroscopy. *Int. Research J. Pharm.* **2012,** 3 (12), 104-108.
[3] Cohen RD, Liu Y, Gong X. Analysis of volatile bases by high performance liquid chromatography with aerosol-based detection. *J. Chrom. A.* **2012,** Mar 16 (1229), 172-9.
[4] Important Considerations Relating to Inverse Gradient for Uniform Response. Thermo Fisher Part Number 4820.4015. Accessed May 18, 2017.
[5] Inverse Gradient for Uniform Response: Quick Installation Guide. Revision A. Thermo Fisher Scientific Part Number 4823.0200. January 2012.

ACKNOWLEDGEMENTS

Susanne Fabel provided the USP standards and developed the ternary method.
Carstel Paul supported data analysis and experimental design.
Paul Gamache and Zhen Long gave technical advice during the workup of data.

TRADEMARKS/LICENSING

ThermoFisher SCIENTIFIC