Quantification of Drug Metabolites in Early-Stage Drug Discovery Testing

Christopher Crafts, Bruce Bailey, John Waraska, Ian Acworth
Thermo Fisher Scientific, Chelmsford, MA, USA

Thermo SCIENTIFIC

Overview

Purpose: The use of a high efficiency UHPLC column and charged aerosol detection were evaluated for the quantification of drug metabolites.

Methods: A 2.1 × 250 mm 2.2 µm UHPLC C18 column with a simple water/acetonitrile gradient was used to compare the performance of charged aerosol detection vs. traditional UV detection techniques.

Results: A more universal response for the majority of the drugs and their metabolites was achieved by charged aerosol detection with an inverse gradient when compared to traditional UV techniques. The reproducibility and sensitivity of the two detectors were similar. Charged aerosol detection is an important complementary technique to those already being used during the *in vitro* drug development stage of testing.

Introduction

Due to new regulations, the identification and quantification of drug metabolites in early-stage drug development testing has become an essential part of the drug development process. Analytical systems typically use reversed phase HPLC or UHPLC with diode array and high resolution mass spectrometry to detect drug metabolites. For many samples however, differences in UV absorbance or inability to ionize can lead to incorrect estimation of metabolite levels. Unfortunately, this over- or under-estimation is typically only revealed at later stage testing when radiochemical approaches are used. In late-stage testing, differences in metabolite quantity can be detrimental to the acceptance process and extremely costly to rectify. Presented here is an orthogonal technique that uses an inverse gradient with the Thermo Scientific Dionex Corona ultra RS charged aerosol detector (CAD™) incorporated into the UV/MS system to overcome most quantification issues.

Charged aerosol detection is a highly sensitive universal technique that offers near uniform response for non-volatile analytes.[1] Like with all nebulization-based techniques, analyte response changes during gradient elution due to changes in nebulization efficiency. This can be rectified by using solvent compensation or an inverse gradient after the analytical column but before the detector.[2] Another challenge with the analysis of drug metabolites is the need for a fast and robust method capable of resolving the API and metabolites for an array of compounds. In early-stage development it is often not possible to optimize HPLC conditions or detector settings from sample to sample. For this reason a long UHPLC column with an acidic water/acetonitrile gradient was chosen for the analysis. The 2.2 µm 250 mm column has sufficient resolving capacity even with injections volumes of 30 µL.

Previous work highlighted the use of charged aerosol detection for the analysis of buspirone and erythromycin and their metabolites without inverse gradient compensation.[3] Although both the Corona™ ultra RS™ charged aerosol detector and low wavelength UV detector showed similar results, late eluting analytes typically showed an increase in response when measured by the CAD. In later studies, this response issue was overcome using an inverse gradient.[4] For the work presented in this poster, an inverse gradient was used for all analyses, thereby keeping solvent composition to the CAD constant and analyte response uniform. In the first part of the study, several parent drugs and metabolite standards were prepared at equal mass quantities and were analyzed using a CAD and an UV detector. The sensitivity and consistency of response were compared for several compounds including acetaminophen and its metabolites. In the second part of the study, drugs were incubated with liver microsomes and then evaluated for mass balance (pre vs. post incubation). The reproducibility and sensitivity of the Corona ultra RS is discussed for the analysis of standards and *in vitro* samples.

Methods

Sample Preparation

Samples were provided already prepared by a major pharmaceutical company for evaluation of the UHPLC/CAD platform. This included standards for active pharmaceutical ingredients (APIs), acetaminophen, clozapine, efavirenz and ranitidine, and metabolites which were prepared at 10 µg/mL in acetonitrile/water solution. Ten micromolar clozapine pre- and post-incubation in rat liver microsomes were also included. The samples were extracted with acetonitrile prior to analysis.

Liquid Chromatography

System:	Thermo Scientific Dionex UltiMate 3000 RSLC system equipped with: DGP-3600RS, WPS-3000TRS, TCC-3000RS, DAD-3000RS, Corona ultra RS
Column:	Thermo Scientific Acclaim RSLC 120 C18, 2.1 × 250 mm, 2.2 µm
Mobile Phase A:	0.1% Formic acid in DI water
Mobile Phase B:	0.1% Formic acid in acetonitrile
Flow Rate	0.75 mL/min pumps left and right.
Gradient Conditions Analytical:	5% B hold 0.1 min, 95% B in 10 min hold for 2, return and equilibrate for 4.
Gradient Conditions Inverse:	95% B hold 1.1 min 5% B in 10 min hold for 2, return.
Injection Volume:	10 µL for standards, 30 µL for microsome samples.
CAD settings:	Filter: 3 Nebulizer Temp On at 25 ˚C Power Function Value 1.0 Flow Diverted 0.6 – 1.1 minutes

Mass Spectrometry

Mass Spectrometry was not used for this evaluation, see discussion of microsome results.

Data Analysis

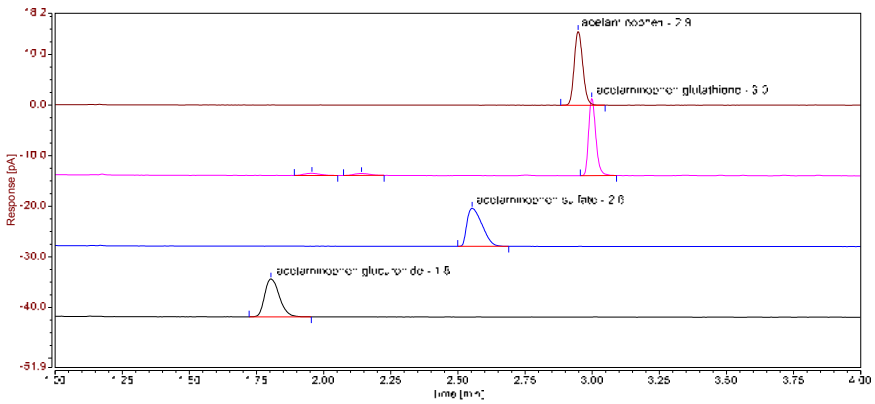
Thermo Scientific Dionex Chromeleon 7.1 SR.1 Chromatography Data System (CDS) was used for all data collection and processing.

Results

Analysis of 10 µg/mL Standards

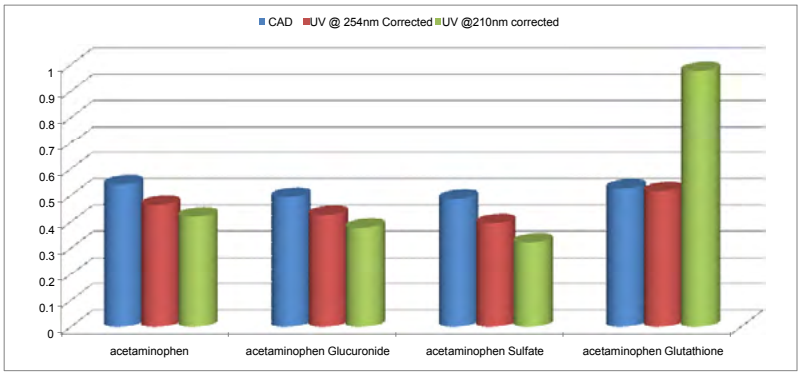
The major metabolites of interest vary depending on the API and its metabolism in the body. In addition to the *in vitro* incubation with microsomes or hepatocytes some compounds undergo further testing for bioactivation and idiosyncratic toxicity using a glutathione conjugation assay.[5] The adduct formation often significantly increases molecular weight of the compound from the API and can have varying impact on the UV absorbance depending on the conjugate and its UV chromophore. To evaluate this problem acetaminophen-glutathione conjugates were prepared and analyzed at concentrations of 10 µg/mL on the UHPLC system with UV (at 210 and 254 nm) detection and charged aerosol detection. Figure 1 shows the overlaid chromatogram of the API and three metabolites by charged aerosol detection.

FIGURE 1: Overlay of acetaminophen and three metabolites at 10 µg/mL (100 ng on-column) measured by charged aerosol detection.



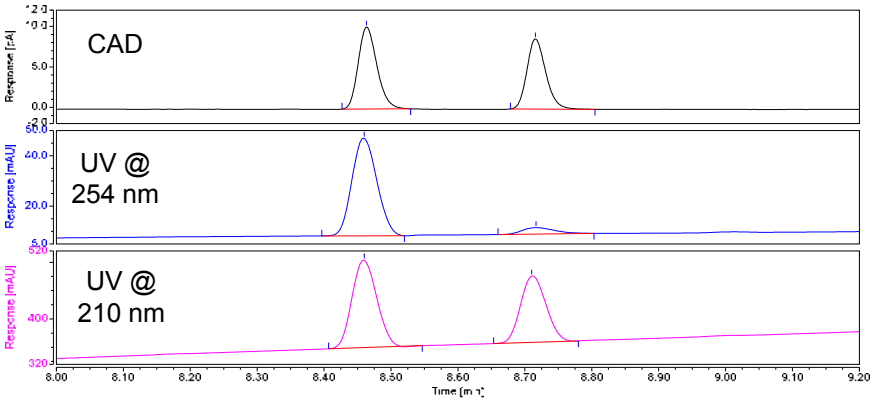
A comparison of the peak areas obtained by the CAD (Figure 1) to those from the two UV wavelengths is presented in Figure 2. Charged aerosol detection is mass sensitive and therefore no data manipulation is required as equal masses of each metabolite were analyzed. UV detection, on the other hand is concentration dependent and will show changes in molar concentration due to differences in MW between the different metabolites. In Figure 3, UV area values are presented corrected for concentration. The results show the consistency of response with charged aerosol detection with a RSD across the four areas of only 5.1%. This is in contrast to the 11.5 and 58% RSD values obtained for the UV at 254 and 210 nm, respectively.

FIGURE 2: Response area for charged aerosol detection and the two UV channels for 10 µg/mL samples of acetaminophen and three metabolites. (UV areas are raw area/MW * 10 to correct for MW and to scale properly).



The results using the specific UV wavelength of 254 nm for acetaminophen showed acceptable deviation levels in this case. However, low wavelength UV which typically offers a more universal or consistent response and is the preferred wavelength for these experiments had a response deviation of 58%. Even in cases were the response deviation is more in line, the sensitivity of low wavelength UV is often insufficient, as was the case for the analysis of ranitidine. Figure 3 illustrates the analysis of 8-hydroxyefavirenz using the same detection parameters. In this case, two peaks were observed by all three detection techniques. The charged aerosol detector trace on top and the UV at 210 nm trace on the bottom are in close agreement to the relative abundance, with peak 1 being 54% by charged aerosol detector and 58 % by UV at 210 nm. However the middle trace using UV at 254 nm has peak 1 with a relative abundance of 92%. Such discrepancies would be very difficult to resolve by UV alone without the use of authentic standards for each peak. Data agreement of the CAD with one of the two other UV results provides sufficient confirmation and speeds up the analysis without need for further investigation or the use of authentic standards.

FIGURE 3: Overlay of chromatograms for analysis of 8-hydroxyefavirenz by charged aerosol detection and UV detection.



Microsome Samples

The goal in early-stage drug development is to obtain *in vitro* results that are similar to the *in vivo* results in later stage testing. It is often not the expectation of a DMPK chemist to obtain 100% recovery or accuracy of the results in this early-stage testing. General practice requires accuracy levels of ~ 20% of the true value to allow confidence in the technique. While this value is fairly large, no single technique is able to deliver these results for every analyte with the sensitivity and turnaround time needed. This is the main reason why a more complex system with multiple detection techniques is required. As presented in our previous report,[3] this approach would normally require a high resolution MS to identify the metabolites. The MS is used in parallel with the UV/charged aerosol detection using a split of 90% post UV (150 µl flow to the MS). However, as the metabolites were already identified and provided for the current study, the MS is not required. To illustrate the power of this approach, data for the analysis of the antipsychotic medication clozapine (10 µM) are presented in Figure 4.

FIGURE 4: Metabolism of clozapine by rat liver microsomes. Data from charged aerosol detection: Top – pre-incubation (T=0); Bottom – post-incubation (T=60).

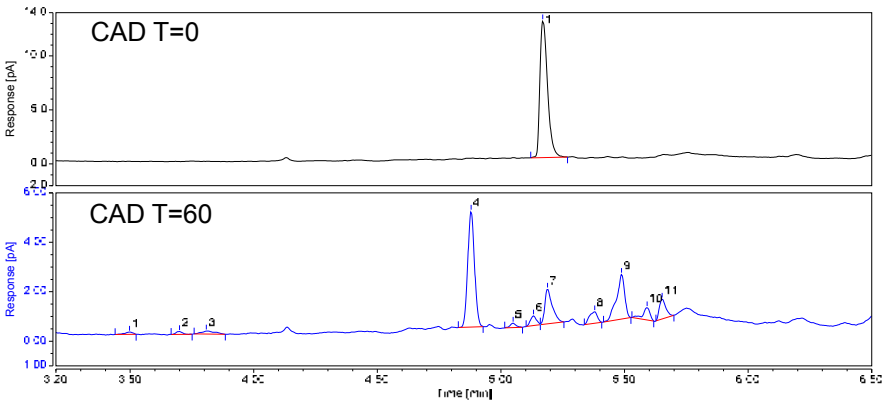


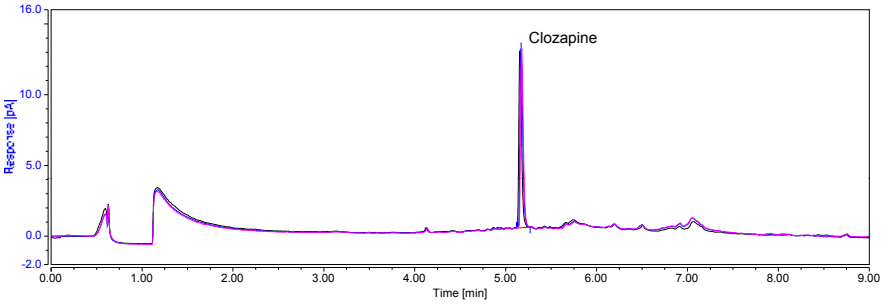
Table 1: Comparison of Area results pre- and post-incubation for clozapine sample.

Incubation Point (Minutes)	Total Raw Area Value		
	CAD	UV @ 254	UV @ 210
T= 0	0.4539	4.677	6.6849
T= 60	0.3574	2.9771	4.3853
% Recovery	79%	64%	66%

Following metabolism by rat liver microsomes, the clozapine peak was significantly decreased, whereas the two major metabolites, n-oxide clozapine (peak 4) and n-desmethyl clozapine (peak 9) significantly increased. The peaks not present in the matrix blank or in the non-incubated sample were used to calculate an area recovery for all three detection techniques. The results using charged aerosol detection were close to the expected results as shown in Table 1, with a 79% area recovery.

Sensitivity and reproducibility are important factors when weighing the effectiveness of quantification techniques for drug metabolites. Figure 5 shows an overlay of three injections of the clozapine sample at T=0. The relative standard deviation for the clozapine peak in the matrix for 3 interlaced injections was 1.4% by the CAD and 1.3% by UV @ 210 nm. The results for the residual clozapine in the T=60 minutes for three interlaced injections (data not shown) was 2.4% with an average signal-to-noise (S/N) of 14/1. The results for the same injections by UV at 210 nm was 4.2% with a S/N of ~ 20/1. The estimated mass on-column for the residual clozapine in the T=60 minute sample is 7 ng on-column. Charged aerosol detection results were still in line with those for the UV technique even at these low levels (near the limit-of-detection) with the added benefit of the near universal response for the non-volatile API and metabolites.

FIGURE 7. Overlay of three interlaced injections of clozapine pre-incubation samples obtained with charged aerosol detection.



Conclusion

- The inclusion of the Corona ultra RS detector as part of a UHPLC/UV/MS system provides another piece of valuable information to help confirm or quantify drug metabolites in early-stage testing.
- The addition of an inverse gradient post-column prior to the CAD and MS detectors is not required but improves the inter-analyte response of these nebulizer-based detectors.
- The sensitivity and reproducibility of the technique was sufficient to analyze the major and minor metabolites formed when clozapine (10 µM) was incubated with rat liver microsomes. Charged aerosol detection is an important complementary technique to those already being used during the *in vitro* drug development stage of testing.
- When analyzing compounds at specific wavelengths, charged aerosol detection helps confirm UV absorbance characteristics of APIs and their metabolites.
- Charged aerosol detection, while not a stand-alone solution, provides additional data to make accurate interpretations of *in vitro* data without excessive cost or time requirements.

References

1. Górecki, T.; Lynen, F.; Szucs, R.; Sandra, P. Universal Response in Liquid Chromatography Using Charged Aerosol Detection. *Anal. Chem.* **2006,** *78,* 3186–3192.
2. DeLand P.; Waraska J.; Crafts C.; Acworth I.; Steiner F.; Fehrenbach T. Improving the Universal Response of Nebulization-Based UHPLC Detection. *LC/GC.* **2011,** April Supplement 45-49.
3. Cai, H.; Crafts, C.; Ramanathan, R.; Humphreys, W.; Bailey, B.; Josephs J.; A Novel Detection Technology: Charged Aerosol Detection (CAD) Coupled with HPLC,UV, and LTQ-Orbitrap MS for Semi-Quantitation of Metabolites in Drug Discovery Metabolism Studies, ASMS oral presentation, Salt Lake City, May, **2010**.
4. Crafts, C.; Plante, M.; Bailey, B.; Malek, G.; Neely, M. Use of Charged Aerosol Detection as an Orthogonal Quantification Technique for Drug Metabolites in Safety Testing (MIST). Eastern Analytical Symposium poster, **2011**.
5. Nedderman, A., Metabolites in Safety Testing: Metabolite Identification Strategies in Discovery and Development. *Biopharm. Drug Dispos.* **2009,** *30*, 153-162.

www.thermofisher.com/dionex

U.S./Canada	(847) 295 7500	**Denmark**	(45) 36 36 90 90	**Sweden**	(46) 8 473 3380	**India**	(91) 22 2764 2735
Brazil	(55) 11 3731 5140	**France**	(33) 1 39 30 01 10	**Switzerland**	(41) 62 205 9966	**Japan**	(81) 6 6885 1213
Austria	(43) 1 616 51 25	**Germany**	(49) 6126 991 0	**United Kingdom**	(44) 1276 691722	**Korea**	(82) 2 2653 2580
Benelux	(31) 20 683 9768	**Ireland**	(353) 1 644 0064	**Australia**	(61) 2 9420 5233	**Singapore**	(65) 6289 1190
	(32) 3 353 42 94	**Italy**	(39) 02 51 62 1267	**China**	(852) 2428 3282	**Taiwan**	(886) 2 8751 6655

Thermo SCIENTIFIC

Part of Thermo Fisher Scientific

PN70036_E **09/16S**