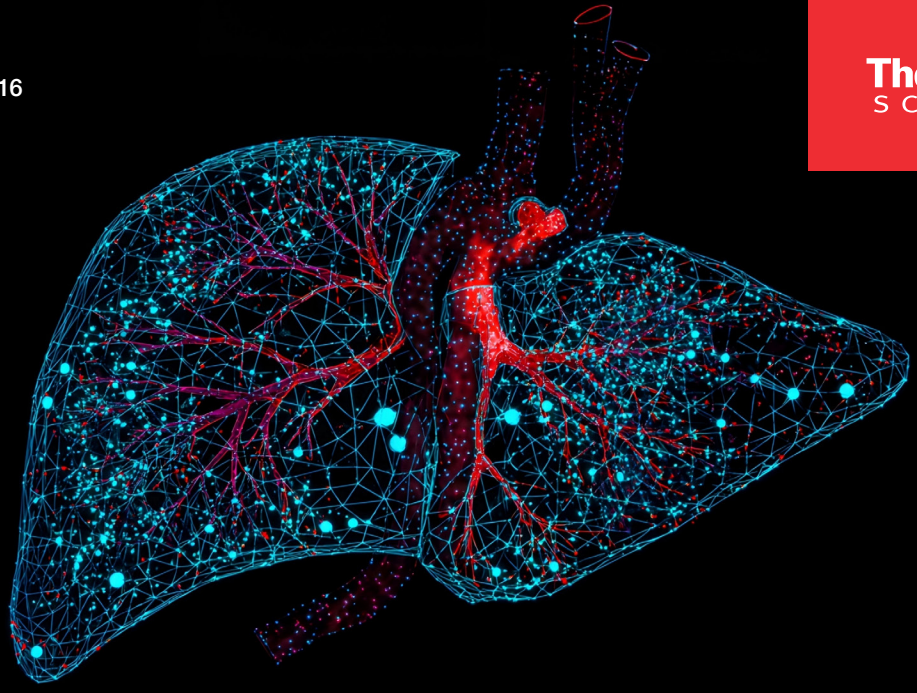
Application note | 004316



Pharma

Early-stage drug metabolite quantitation without radiolabels

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Keywords

Vanquish CAD, Vanquish Charged Aerosol Detector P series (CAD P), DMPK, MIST, microsomes, cytochrome P-450, drug metabolites in safety testing

Application benefits

- Method allows quantitation of metabolites without radiolabeling.
- Method is ideal for quantitation of metabolites early in the drug development process, without the need for reference standards for each component.
- Quantitation of the parent compound and all nonvolatile metabolites down to nanogram levels is achieved due to the wide dynamic range of the Thermo Scientific™ Vanquish™ Charged Aerosol Detector P series.
- The diverter valve allows the user to switch between CAD and the MS for comparison purposes.

Goal

To quantify clozapine and its metabolites in liver microsomes using charged aerosol detection without the need for radiolabeling or reference standards.

Introduction

Early prediction of drug metabolism by cytochrome P-450 in the liver is key to the drug development process. Significant formation of toxic metabolites will force any promising candidate out of the development pipeline. The key step is *quantitation* to determine if a substance is present at levels above a certain threshold of concern. Any peak greater

than the threshold must be identified in extensive tests with mass spectrometry (MS) and sometimes even tested for toxicity in animal models. The critical quantitation step is complicated by the fact that standards do not usually exist for new, unexpected metabolites.

The gold standard for metabolite quantitation is the use of radiolabeled substrates, such that the metabolites are radioactive and can be quantified using a radiometric detector. When labs choose to forgo the time-consuming synthesis of expensive radioactive substrates and the preparation of the lab space to handle radioactive substances, several options for quantification exist. Quantitation by high performance liquid chromatography (HPLC) with MS without standards for each peak is complicated because ionization efficiency, and therefore MS response, depends on chemical structure. Quantitation by ultraviolet (UV) detection without standards can also yield inconsistent results because detector responses vary based on the structure of the chromophore. Quantitation by evaporative light scattering detection (ELSD) is possible without a standard, but the methods are often insufficiently sensitive, and response depends on chemical properties such as refractive index.

Quantitation by charged aerosol detector (CAD) provides the most consistent response across all nonvolatile and some semivolatile analytes of all HPLC detection techniques.

The detector works by evaporating the solvent, charging the remaining analyte particles in proportion to their mass, and measuring the resulting electrical signal, providing a near-universal response that is independent of light scattering (unlike ELSD). An inverse gradient compensation (IGC) was implemented to ensure a constant mobile phase composition at the detector, despite the gradient, and to maximize the CAD quantitation performance. The performance of the method presented here was evaluated using clozapine, whose metabolites retain the UV chromophore and for which both the UV and CAD estimations are quite accurate. This analysis can easily be transferred to a substance for which the metabolites have no good UV chromophore and for which the chromophore-independent CAD quantitation shows advantages over UV. During the study, the application was optimized to take advantage of improvements in the Vanquish Charged Aerosol Detector P series (CAD P). The changes made included use of the diverter valve to switch the salt-rich matrix to waste to protect the detector from contamination, collection of data simultaneously on four channels with different power values (PVs) to optimize the system for a linear response, use of coupling mode to bring the temperature of the charging-detection module down to 5 °C above the evaporation tube temperature, and use of the diverter valve to switch between the CAD and the MS.

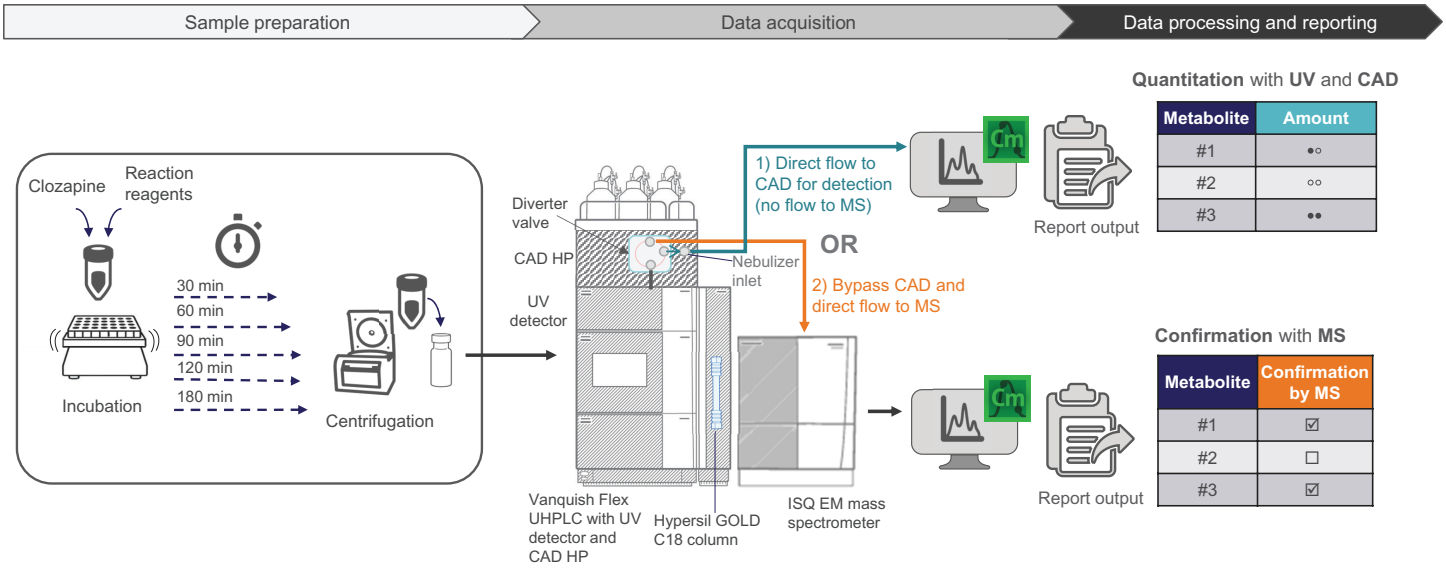

Figure 1. Schematic illustrating the workflow for clozapine metabolite quantitation and confirmation. Data acquisition was performed using a Thermo Scientific™ Vanquish™ Flex UHPLC System equipped with a Thermo Scientific™ Vanquish™ Diode Array Detector F (DAD FG), Vanquish Charged Aerosol Detector HP (CAD HP), and Thermo Scientific™ ISQ™ EM Mass Spectrometer (MS), using a Thermo Scientific™ Hypersil GOLD™ Column. The UHPLC system allows, by switching the valve in the CAD HP, either the quantitation of the metabolites by UV and CAD or the confirmation by MS. Data were acquired and processed using the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS).

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification (Cat. No. 50136149)
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (Cat. No. A955)
- Fisher Scientific™ Methanol, Optima™ LC/MS grade (Cat. No. A456)
- Thermo Scientific™ Ammonium bicarbonate, HPLC grade 99%+ (Cat. No. 446232500)
- Fisher Scientific™ Formic acid, Optima™ LC/MS grade (Cat. No. A117-50)
- Gibco™ Human microsomes from liver, 50 donor pool (Cat. No. HMMCPL)
- Thermo Scientific™ Clozapine, 97%, CAS 5786-21-0 (Cat. No. J61583.MB)

Sample handling

- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipette: 100–1,000 µL (Cat. No. 4641100N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipette: 10–100 µL (Cat. No 4641070N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipette: 1–10 µL (Cat. No 4641030N)
- Fisherbrand™ Mini Vortexer (Cat. No 14-955-152)
- Thermo Scientific™ Digital Heating Shaking Drybath (Cat. No 88880028)
- Thermo Scientific™ Orion™ 3 Star pH Benchtop Meter (Cat. No 13-644-928)
- Fisherbrand™ accuSpin™ 8C Small Benchtop Centrifuge (Cat. No 75-008-821)
- Fisherbrand™ Microcentrifuge Tubes with Locking Snap Cap (Cat. No 14-666-326)
- Thermo Scientific™ SureSTART™ 0.2 mL Amber TPX Screw Microvial with Glass Insert, Level 3 (Cat. No 60180-1655)
- Thermo Scientific™ SureSTART™ Red PTFE/White Silicone/Red PTFE Screw (AVCS™), Level 3 (Cat. No 6PSC9TST)

Instrumentation

Vanquish Flex UHPLC system consisting of:

- Thermo Scientific™ Vanquish™ System Base (Cat. No. VF-S01-A)
- Thermo Scientific™ Vanquish™ Dual Gradient Pump F (Cat. No. VF-P32-A)
- Thermo Scientific™ Vanquish™ Split Sampler F (Cat. No. VF-A10-A)
- Thermo Scientific™ Vanquish™ Column Compartment H (Cat. No. VH-C10-A)
- Thermo Scientific™ Vanquish™ Diode Array Detector F (Cat. No. VF-D11-A)
- Thermo Scientific™ Vanquish™ Standard Flow Cell, 13 µL, path length 10 mm, SST (Cat. No. 6083.0510)
- Thermo Scientific™ Vanquish™ Charged Aerosol Detector HP (Cat. No. VH-D21-A)
- Thermo Scientific™ ISQ™ EM mass spectrometer (Cat. No. ISQEM-ESI)
- Thermo Scientific™ Viper™ TQ Capillary Kit for Vanquish Inverse Gradient Systems (Cat. No. 6036.2010A)

Sample preparation

For the digestion reaction, 50 mM ammonium bicarbonate, pH 7.4, 2 mg/mL clozapine, 20 mg/mL microsomes, and 20 mM NADPH were required. Ammonium bicarbonate buffer was prepared by adding 0.998 g ammonium bicarbonate to a graduated cylinder, filling to the 250 mL mark with water, and adding 70 µL formic acid. The pH was 7.4, although it increased slowly with exposure to air because CO_2 evaporates faster than ammonia. Ammonium bicarbonate buffer was prepared freshly directly before carrying out the reaction to ensure that the pH was correct. Clozapine was dissolved in acetonitrile at 2 mg/mL. NADPH was prepared in ammonium bicarbonate buffer immediately before the digestion reaction, and the microsomes were thawed on ice. The digestion reaction matrix consisted of 193 µL bicarbonate buffer, 2 µL of clozapine solution, and 5 µL microsomes in a 0.5 mL microcentrifuge tube. As shown in Figure 1, reactions were started by the addition of 10 µL NADPH and incubated for 30, 60, 90, 120, and 180 min at 37 °C with gentle agitation. Samples were vortexed and centrifuged for 10 min at 14,000 RPM. The supernatant was pipetted into microvials for analysis.

Chromatographic conditions

The chromatographic conditions are detailed in Table 1. The inverse gradient kit and fluidic wizard were used to calculate an IGC offset of 538.50 µL using the "keep solvent composition" calculation mode.

The bypass position of the diverter valve, used here to switch between CAD and MS (Figure 1), must be activated in the Instrument Configuration Manager. When the valve position is set to "bypass," both the "nebulizer" and "waste" lights are on in the status keypad on the front of the module. The valve was switched from waste to bypass position after 1.6 minutes, diverting the flow to the MS.

MS settings

Methods for the ISQ EM mass spectrometer were created using the "easy" default settings in the method editor. The MS was operated in electrospray ionization mode (HESI) with positive polarity (Table 2). Two alternating Full MS scans were run in the "scan mode" method type with the detection ranges set to *m/z* 90–400 and *m/z* 400–1,000.

Table 1. Chromatographic conditions.

Column	Thermo Scientific™ Hypersil GOLD™ C18 150 x 2.1 mm, 1.9 µm column (Cat. No. 25002-152130)
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
	Flow rate [mL/min] from PumpLeft and PumpRight / **Time [min]** / **B [%]** 0.5 / 0.00 / 20 0.5 / 4 / 55.8 0.5 / 4 / 100 0.5 / 7.5 / 100 0.5 / 7.5 / 20 0.5 / 11 / 20 IGC: All steps delayed by 1.53 min. Inverse gradient between 100% and 64.2% B.
Column temperature	30 °C still air mode, 30 °C active preheater
Autosampler temperature	6 °C
Needle wash solution	10/90 water/isopropanol v/v
Needle wash mode	AfterDraw, 6.0 s, 100.0 µL/s, 500 µm puncture offset, needle height 1,000 µm
Injection volume	5 µL
DAD settings	Wavelengths: 210, 230, 260, 300 nm, Bandwidth: 4 nm, Data collection rate: 10 Hz, Response time: 0.5 s, 3D field: from 190 to 400 nm with 4 nm bunchwidth
CAD settings	Evaporation temperature: 25 °C, Filter constant: 5 Hz, Data collection rate: 10 Hz, Coupling mode: On, Diverter valve switch: from nebulizer to waste: 0.3 min; from waste to nebulizer: 1.6 min; from nebulizer to waste: at end of method, Power value (PV): 1.5 for CAD_1, 1.8 for CAD_2, 1.65 for CAD_3, 1.95 for CAD_4

Table 2. Instrument and scan settings for the mass spectrometer.

Ionization mode	HESI
Polarity (Spray voltage)	Positive (+ 3,000 V)
Scan mode	*m/z* 90–400 (low), *m/z* 400–1,000 (high)
Total scan time	0.208 s
Scan time	0.1 s
CID voltage	10 V
Vaporizer temperature	282 °C
Ion transfer tube temperature	300 °C
Gas flow pressure	Sheath gas: 49.9 psig Auxiliary gas: 5.7 psig Sweep gas: 0.5 psig

Chromatography Data System

The Thermo Scientific™ Chromeleon™ 7.3.2 Chromatography Data System (CDS) was used for data acquisition and analysis. The CAD P Series driver is compatible with Chromeleon 7.3.2 CDS and later.

Data analysis

Signal to noise (S/N) was calculated based on a 0.5 min window around the retention time of the peak of interest from a chromatogram of an injection of the mobile phase starting conditions. The limit of quantification was set at the concentration of the smallest standard that achieved a S/N ratio of ≥10. The legacy power function value (PFV) is convertible to a power value (PV) by multiplying PFV by 1.5. For more information on PV refer to Technical Note 003816.[1]

Results and discussion

The study focused on quantifying clozapine and its metabolites in liver microsomes using UV and CAD, while MS was used to confirm the metabolites known from literature.[2]

Method optimization

Five different incubation times were tested as part of sample preparation optimization. The relative standard deviation (RSD) for the clozapine peak area by CAD after 180 min incubation was 4.2% (n=3).

The impact of the evaporation temperature (EvapT) between 25 °C and 50 °C was investigated to optimize CAD performance. Although both APIs are nonvolatile, a CAD EvapT of 25 °C was chosen to improve the response of any potential semivolatile metabolites. The EvapT can be set as low as five degrees above room temperature (for reference, the laboratory temp was 18 °C). The difference in the background current between 25 °C and 50 °C EvapT was negligible for this application, so there was no need to use a higher EvapT to reduce background from the mobile phase (Figure 2). The background current always uses the power value of the CAD_1 channel, which was 1.5 (legacy PFV 1.0) for this application.

Analysis of clozapine

Calibration and limit of quantitation

Four data channels were collected using CAD at power values of 1.5, 1.65, 1.8, and 1.95 (legacy power function values 1.0, 1.1, 1.2, and 1.3), but the concentration range was about two orders of magnitude and perfectly in the quasi-linear area of the CAD response (Figure 3), so that a linear fit showed minimal residuals of below 15% on all four channels (example for PV 1.5 shown in Figure 4). The coefficients of determination (r^2) were all ≥0.998 and were unhelpful for determining goodness-of-fit (Table 3). A power value of 1.5 was chosen based on the residual plots. The peak heights at low ng/µL concentrations allowed for a limit of quantitation (LOQ) of 0.67 ng/µL or 3.4 ng on column for the CAD. The S/N ratio for the 3.4 ng on column sample with the UV detector at 260 nm was 80.6, and the calculated LOQ for the UV detector was 0.45 ng on column (equals 0.09 ng/µL). CAD limits of quantification cannot be extrapolated, so the LOQ was taken as the lowest calibration standard. The highest standard was not used for the MS calibration curve to improve the quantitation, and a quadratic fit was used.

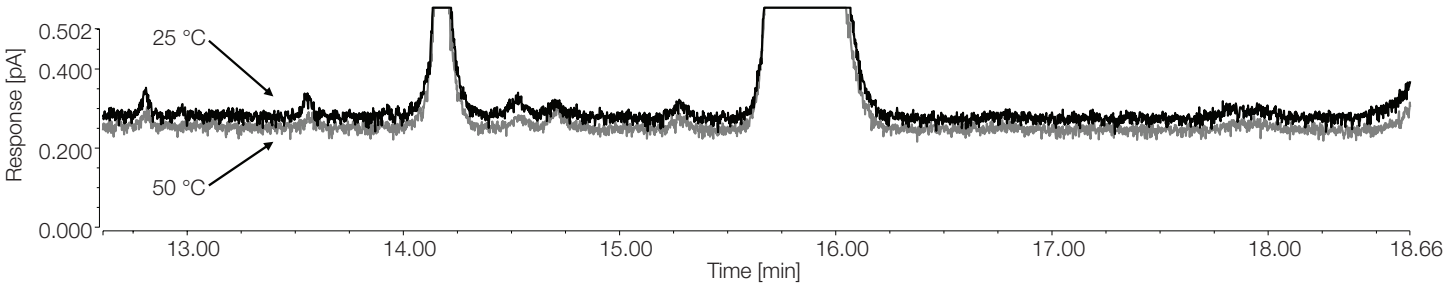

Figure 2. Background current at 25 °C (black) and 50 °C (gray) for the EvapT.

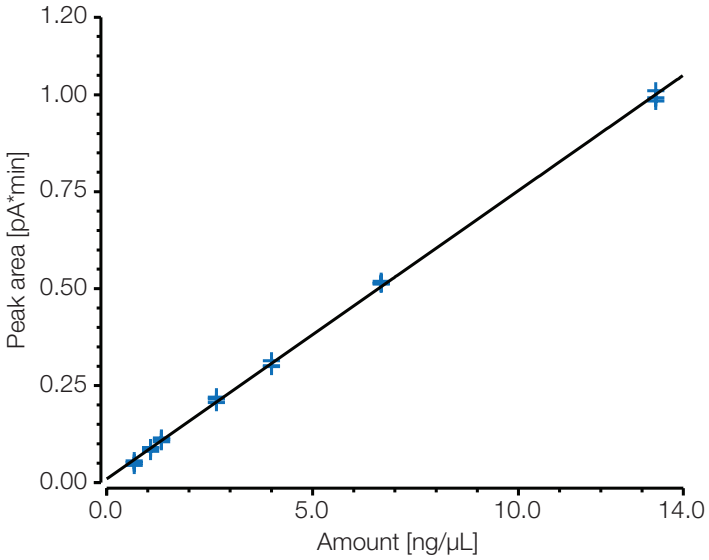

Figure 3. Linear response curve for clozapine with CAD (PV 1.5) from 0.67 ng/µL (3.4 ng on column) to 13.33 ng/µL (67 ng on column).

Table 3. Coefficients of determination (r^2) for the linear fit of the clozapine response for all four CAD channels.

Channel	Power value	Coefficient of determination (r^2)
CAD_1	1.5	0.9992
CAD_2	1.65	0.9987
CAD_3	1.8	0.9981
CAD_4	1.95	0.9980

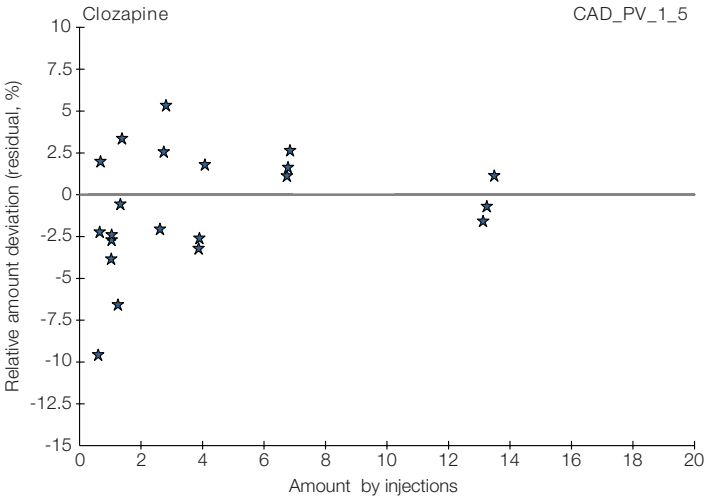

Figure 4. Plot of relative amount deviation in percentage of each injection in the calibration curve from the linear fit for channel CAD_1, PV = 1.5 (legacy PFV = 1.0).

Quantitation of metabolites

Three detection techniques were used in this study. While the UV detector and the CAD were coupled in series, the diverter valve in the CAD was used to switch between nebulizer position (CAD) or bypass position (MS). The MS was used for qualitative purposes to confirm metabolite presence. In addition, the diverter valve in the CAD was used to direct the first 1.6 min into waste to remove the digestion reaction matrix. The chromatograms of all three detectors are shown in Figure 5.

The complementary techniques of UV and CAD were used to quantify the microsome samples. While the CAD is a mass sensitive and universal detector, the UV is concentration-dependent and shows a different response due to varied chromophoric activities between the different metabolites. Therefore, typically quantitation with CAD can be done with a single standard, whereas UV quantitation requires standards for each compound. As they are not available, the UV quantitation is done by using clozapine as the reference compound in calibration for all metabolites. This leads to an over- or underestimation in quantitation. Quantitation results for the total peak area are shown in Table 4 and quantitation results for the metabolites are shown in Table 5.

Table 4. Total peak area results for CAD, UV (260 nm), and MS detectors at incubation times of 0 and 180 min and recovery rates for the API clozapine and the metabolite peaks 1-3. Values presented are averaged from 3 injections.

Incubation time	CAD total area (pA*min)	UV total area (mAU*min)
0 min	1.01 (4.0% RSD)	9.49 (0.3% RSD)
180 min	0.94 (4.2% RSD)	8.74 (0.8% RSD)
Recovery	93%	92%

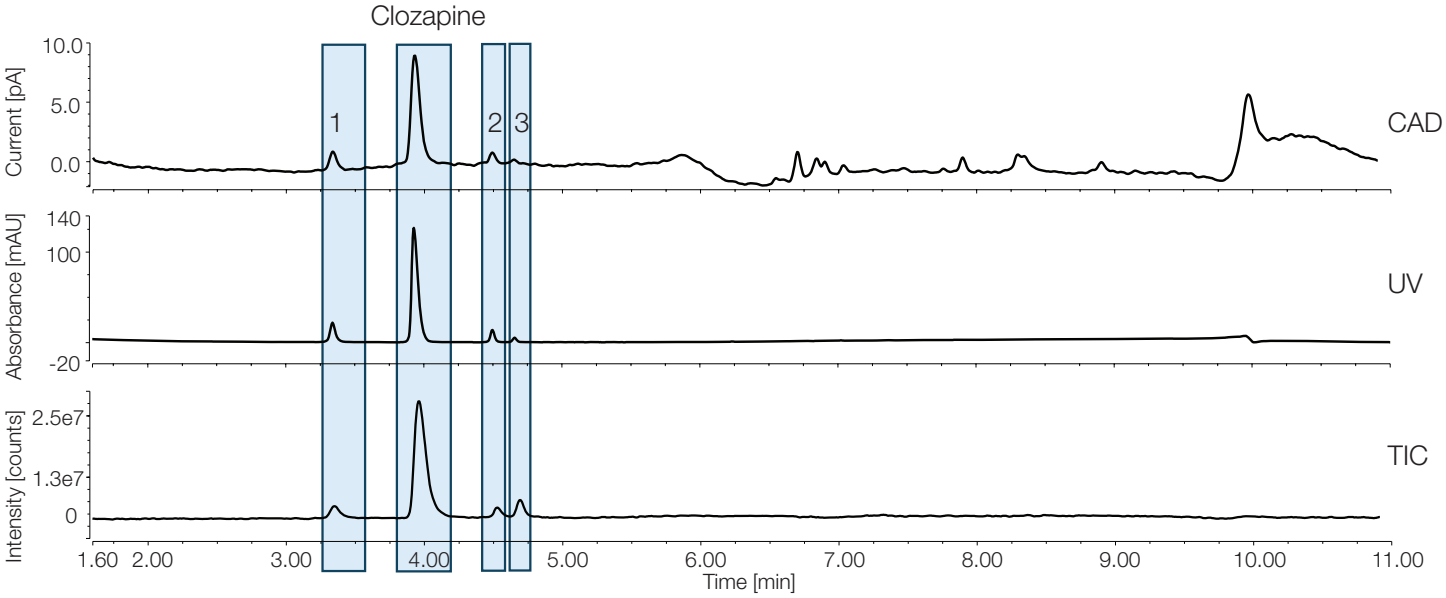

Figure 5. CAD, UV (260 nm), and total ion chromatogram (TIC) scan range *m/z* 90-400 showing the API peak (clozapine) and related metabolite peaks (1-3) after an incubation time of 180 min.

Table 5. Quantitation results for the metabolites at incubation time of 180 min. The LOQ for CAD is 0.67 ng/µL and the LOQ for UV at 260 nm is 0.09 ng/µL.

Metabolite peak number	Retention time [min]	Amount by CAD [ng/µL]	Amount by UV [ng/µL]	Mass (*m/z*)	Tentative assignment
1	3.53	1.52	1.44	313.1	N-demethylclozapine*
2	4.53	0.54	0.66	343.1	clozapine-N-oxide*
3	4.70	0.43	0.21	352.1	NA

*MS confirmation based on known literature data[2]

The UV quantitation of metabolites was based on clozapine calibration because no standards were available for the P450 metabolites, and this quantitation can lead to over- or underestimation of results. Response by CAD is uniform for nonvolatile and some semivolatile substances. To optimize the CAD uniform response, the EvapT of this analysis was set to 25 °C and the coupling mode was used to bring the temperature of the charging-detection module down to 5 °C above the evaporation tube temperature. The percentage recovery was 93% for CAD and 92% for UV. The response by MS was insufficiently uniform for quantitation due to different ionization of the metabolites. For clozapine, the response was uniform for both the CAD and the UV because oxidation did not significantly modify the clozapine chromophore. When the substances have no chromophore or when oxidation greatly affects the chromophore, the analysis can only be performed by CAD, for which the response is independent of molecular structure and properties. The MS was used to identify the metabolites. As shown in Table 5, peak 2 results from the loss of a methyl group, peak 3 results from oxidation of a nucleophilic nitrogen center, and peak 4 is not identified and would require an MS with fragmentation capability that offers fragmentation analysis. The amounts determined by CAD and UV were slightly below the LOQ, which is set only as low as the lowest calibration standard. A calibration standard at 0.2 ng/µL or 0.3 ng/µL would still likely be in the linear region and should be incorporated the next time the analysis is performed.

Conclusion

The presented method advances early-stage drug metabolite quantitation by eliminating the need for radiolabeling and metabolite reference standards. The integrated diverter valve enhances flexibility by enabling automatic switching between CAD for quantitative analysis and MS for qualitative metabolite confirmation without user interaction. While UV detection can be applied using a single calibrant, differences in chromophoric response may result in over- or underestimation. MS provides compound confirmation but is not suitable for single-calibrant quantitation due to variable ionization efficiency. Overall, the CAD offers the most uniform quantitation compared to UV and MS for these kind of applications. The study yielded the following results for the CAD method:

- Optimal results were achieved at incubation time of 180 min, EvapT of 25 °C, and PV of 1.5.
- Limit of quantitation is 0.67 ng/µL or 3.4 ng on column, based on the lowest calibration standard measured.
- Recovery for total peak area of target compounds was 93%.
- Metabolite quantitation results ranged from < LOQ to 1.52 ng/µL.

Reference

1. Amatobi, K. and Lovejoy, K. Thermo Fisher Scientific TN003816: Method transfer and optimization of deoxycholic acid analysis using HPLC-CAD, **2025**. https://documents.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-003816-hplc-vanquish-method-transfer-cad-deoxycholic-acid-tn003816-na-en.pdf
2. 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**.

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