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Nota applicativa

# A Validated Method for the Quantification of Clopidogrel in Human Plasma at the 2.5 pg/mL Level Using Xevo TQD, ACQUITY UPLC H-Class System, and UNIFI Scientific Information System

Jennifer L. Simeone, Paul D. Rainville

Waters Corporation

## Abstract

Clopidogrel is a thienopyridine derivative antiplatelet pro-drug used in the prevention of artherosclerotic events. This application note describes the development of a highly sensitive solid phase extraction and LC-MS/MS assay for the analysis of the pro-drug clopidogrel in human plasma with an assay sensitivity of 2.5 pg/mL.

### Benefits

The analysis of clopidogrel in human plasma was successfully completed using a high sensitivity method.


## Introduction

Clopidogrel is a thienopyridine derivative antiplatelet pro-drug used in the prevention of artherosclerotic events. Following oral administration, the dosed compound undergoes hepatic metabolism giving rise to the active thiol-metabolite and the inactive carboxylic acid metabolite. The inactive metabolite accounts for the majority of circulating clopidogrel-related material in humans, while the active metabolite and unchanged pro-drug are present at very low levels. The mechanism of action is derived from the binding of the active thiol-metabolite to cell receptor P2Y12, irreversibly inhibiting the platelet activation process.[1]

Due to the reactivity of the active thiol-metabolite coupled with the low levels of the unchanged pro-drug, most quantitative studies are based on the circulating levels of the inactive metabolite. This application note describes the development of a highly sensitive solid phase extraction and LC-MS/MS assay for the analysis of the pro-drug clopidogrel in human plasma with an assay sensitivity of 2.5 pg/mL.

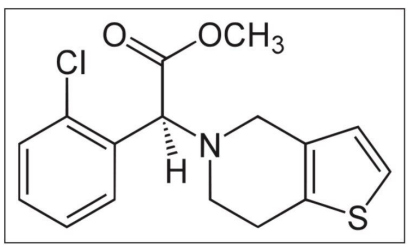


*Figure 1. Structure of clopidogrel.*

## Experimental

### Sample description

Samples were prepared using an Oasis MCX µElution Solid Phase Extraction (SPE) Plate. 350 µL of plasma sample was mixed with 20 µL of internal standard (deuterated clopidogrel) solution and 350 uL of aqueous buffer. The samples were applied to the solid phase extraction plate that was previously conditioned with methanol and aqueous buffer. The sample was washed with an aqueous – methanol solution and eluted with 2 x 25 µL of 5% $NH_4OH$ in 60:40 IPA/ACN, then further diluted with 25 µL of water prior to injection. The analysis was performed on an ACQUITY UPLC H-Class chromatography system. A 10-µL aliquot of the sample was injected onto an ACQUITY UPLC $C_{18}$ 2.1 x 50 mm, 1.7 µm Column. The column was eluted under gradient conditions over 3 min at a flow rate of 600 µL/min. The column effluent was monitored using a Xevo TQD Mass Spectrometer operated in multiple reaction monitoring (MRM) positive ion electrospray mode. The transition 322 → 212 was employed for the clopidogrel and the transition 326 → 216 was employed for the $d_4$ internal standard.

## Results and Discussion

Clopidogrel eluted at a retention time of 2.10 min, as shown in Figure 2. The peak produced by the chromatography system was very symmetrical with a width at the base of 3 seconds. The sharpness of the peak and the symmetrical nature enabled efficient processing and peak integration. The three-minute analysis time allows for a total analysis time of five hours for a 96-well SPE plate, providing at least two plates to be processed per day. The data displayed in Figure 2 illustrates the injection of an extracted plasma blank injection immediately following analysis of the 500 pg/mL standard. This data revealed that there was no discernible carryover in the blank chromatogram. The extremely low carryover exhibited by the ACQUITY UPLC H-Class System allowed the full sensitivity of the Xevo TQD Mass Spectrometer to be exploited.

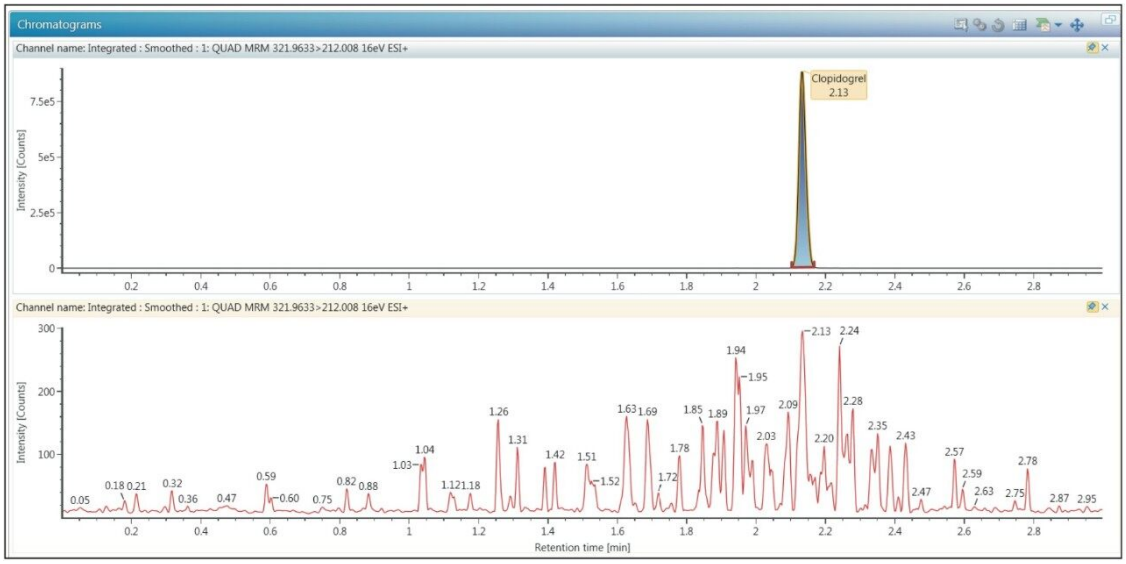


*Figure 2. LC-MS/MS chromatogram of extracted blank and 500 pg/mL = clopidogrel standard.*

The lower limit of quantification (LLOQ) for the assay was determined to be 2.5 pg/mL. The assay was validated with separate 96 well batches on three consecutive days over the range of 2.5 to 500.0 pg/mL. A typical calibration obtained for the assay is shown in Figure 3. The correlation coefficient ranged between 0.9988 and 0.9995 using a 1/x weighted linear regression. The intra-day precision and accuracy validation data is shown in Tables 1, 2, and 3. The validation data shows that the coefficient of variation ranged from 5.8% to 12.2% for the 2.5 pg/mL LLOQ with a bias between -6.9% and -13.6%. For the high QC (300 pg/mL), the coefficient of variation ranged from 1.2% to 2.9% with a bias between 1.4% and 1.6%. The inter-day precision and accuracy data is displayed in Table 4. The coefficient of variation was determined to be 8.5% for the 2.5 pg/mL LLOQ with a bias of -9.4. For the high QC (300 pg/mL), the coefficient of variation was determined to be 2.3% with a bias of 1.5%.

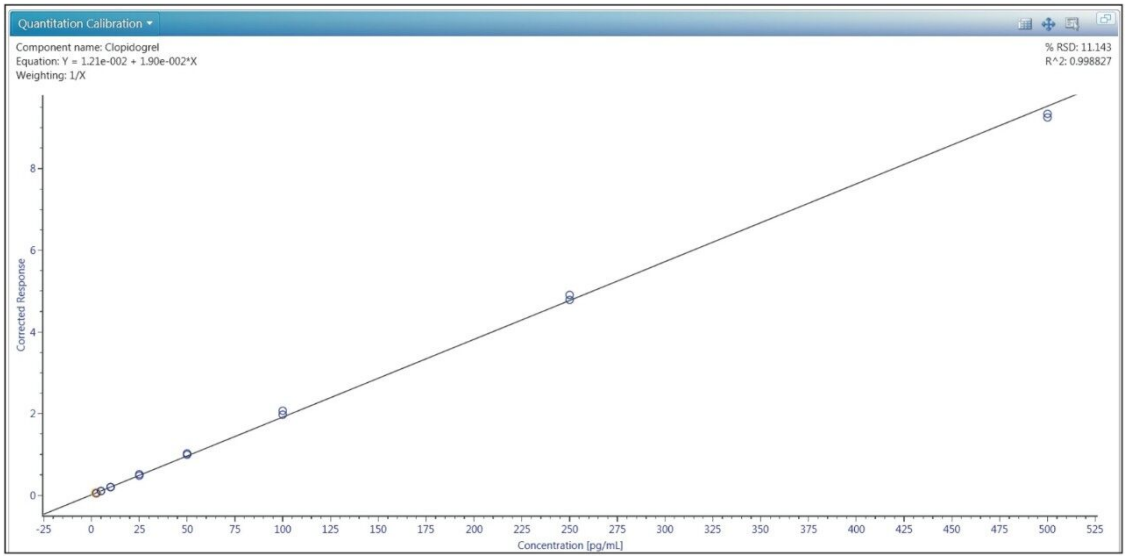


*Figure 3. Representative calibration line for the LC-MS/MS quantification of clopidogrel in plasma.*

| | QC LLOQ 2.5 pg/mL | QC Low 7.5 pg/mL | QC Mid 75 pg/mL | QC High 300 pg/mL |
|---|---|---|---|---|
| | 2.30 | 7.57 | 80.5 | 314 |
| | 2.07 | 6.64 | 79.7 | 307 |
| | 2.30 | 7.38 | 80.6 | 302 |
| | 2.39 | 7.29 | 84.4 | 300 |
| | 1.74 | 7.52 | 81.7 | 292 |
| | * | 7.10 | 77.7 | 313 |
| **Mean** | 2.16 | 7.25 | 80.8 | 305 |
| **St Dev** | 0.263 | 0.343 | 2.223 | 8.38 |
| **%CV** | 12.2 | 4.7 | 2.8 | 2.8 |
| **%Bias** | -13.6 | -3.3 | 7.7 | 1.6 |

*Table 1. Intra-day QC accuracy/precision statistics: day 1.*

**QC LLOQ Rep 6 was a blocked SPE well, no eluent injected.*

| | QC LLOQ 2.5 pg/mL | QC Low 7.5 pg/mL | QC Mid 75 pg/mL | QC High 300 pg/mL |
|---|---|---|---|---|
| | 2.15 | 7.13 | 73.1 | 290 |
| | 2.08 | 7.28 | 76.4 | 316 |
| | 2.32 | 7.19 | 71.5 | 303 |
| | 2.56 | 6.60 | 74.6 | 309 |
| | 2.36 | 7.97 | 76.0 | 302 |
| | 2.27 | 7.24 | 82.9 | 309 |
| **Mean** | 2.29 | 7.24 | 75.8 | 305 |
| **St Dev** | 0.169 | 0.438 | 3.950 | 8.84 |
| **%CV** | 7.4 | 6.1 | 5.2 | 2.9 |
| **%Bias** | -8.4 | -3.5 | 1.0 | 1.6 |

*Table 2. Intra-day QC accuracy/precision statistics: day 2.*

| | QC LLOQ 2.5 pg/mL | QC Low 7.5 pg/mL | QC Mid 75 pg/mL | QC High 300 pg/mL |
|---|---|---|---|---|
| | 2.28 | 7.28 | 77.6 | 304 |
| | 2.23 | 8.04 | 77.0 | 304 |
| | 2.36 | 7.50 | 77.3 | 299 |
| | 2.16 | 7.00 | 76.5 | 306 |
| | 2.54 | 7.02 | 78.2 | 310 |
| | 2.39 | 7.55 | 77.8 | 303 |
| **Mean** | 2.33 | 7.40 | 77.4 | 304 |
| **St Dev** | 0.134 | 0.390 | 0.603 | 3.61 |
| **%CV** | 5.8 | 5.3 | 0.8 | 1.2 |
| **%Bias** | -6.9 | -1.4 | 3.2 | 1.4 |

*Table 3. Intra-day QC accuracy/precision statistics: day 3.*

| | QC LLOQ 2.5 pg/mL | QC Low 7.5 pg/mL | QC Mid 75 pg/mL | QC High 300 pg/mL |
|---|---|---|---|---|
| | 2.30 | 7.57 | 80.5 | 314 |
| | 2.07 | 6.64 | 79.7 | 307 |
| | 2.30 | 7.38 | 80.6 | 302 |
| | 2.39 | 7.29 | 84.4 | 300 |
| | 1.74 | 7.52 | 81.7 | 292 |
| | * | 7.10 | 77.7 | 313 |
| | 2.15 | 7.13 | 73.1 | 290 |
| | 2.08 | 7.28 | 76.4 | 316 |
| | 2.32 | 7.19 | 71.5 | 303 |
| | 2.56 | 6.60 | 74.6 | 309 |
| | 2.36 | 7.97 | 76.0 | 302 |
| | 2.27 | 7.24 | 82.9 | 309 |
| | 2.28 | 7.28 | 77.6 | 304 |
| | 2.23 | 8.04 | 77.0 | 304 |
| | 2.36 | 7.50 | 77.3 | 299 |
| | 2.16 | 7.00 | 76.5 | 306 |
| | 2.54 | 7.02 | 78.2 | 310 |
| | 2.39 | 7.55 | 77.8 | 303 |
| **Mean** | 2.26 | 7.29 | 78.0 | 305 |
| **St Dev** | 0.192 | 0.376 | 3.28 | 6.90 |
| **%CV** | 8.5 | 5.2 | 4.2 | 2.3 |
| **%Bias** | -9.4 | -2.7 | 4.0 | 1.5 |

*Table 4. Inter-day QC accuracy/precision statistics.*

**QC LLOQ Rep 6 was a blocked SPE well, no eluent injected.*

## Conclusion

- A high sensitivity method has been developed for the analysis of clopidogrel in human plasma.
- The assay showed excellent intra- and inter-day precision and accuracy in a three-day validation study.
- The level of quantification was determined to be 2.5 pg/mL with a %CV and bias both considerably below the +/- 20% criteria.
- The carryover was determined to be significantly less than 20% of the LLOQ in an extracted blank, following the injection of a high concentration standard.

## References


1. Pereillo JM, Maftouh M, Andrieu A, Uzabiaga MF, Fedeli O, Savi P, Pascal M, Herbert JM, Maffrand JP, Picard C. Structure and stereochemistry of the active metabolite of clopidogrel. *Drug Metabolism and Disposition*. 2002; 30: 1288-1295.


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