應用手冊

Bioanalysis of Clopidogrel in Plasma: Sub-Picogram Estimation Using the ACQUITY UPLC System, Xevo TQ-S MS, and MassLynx Software

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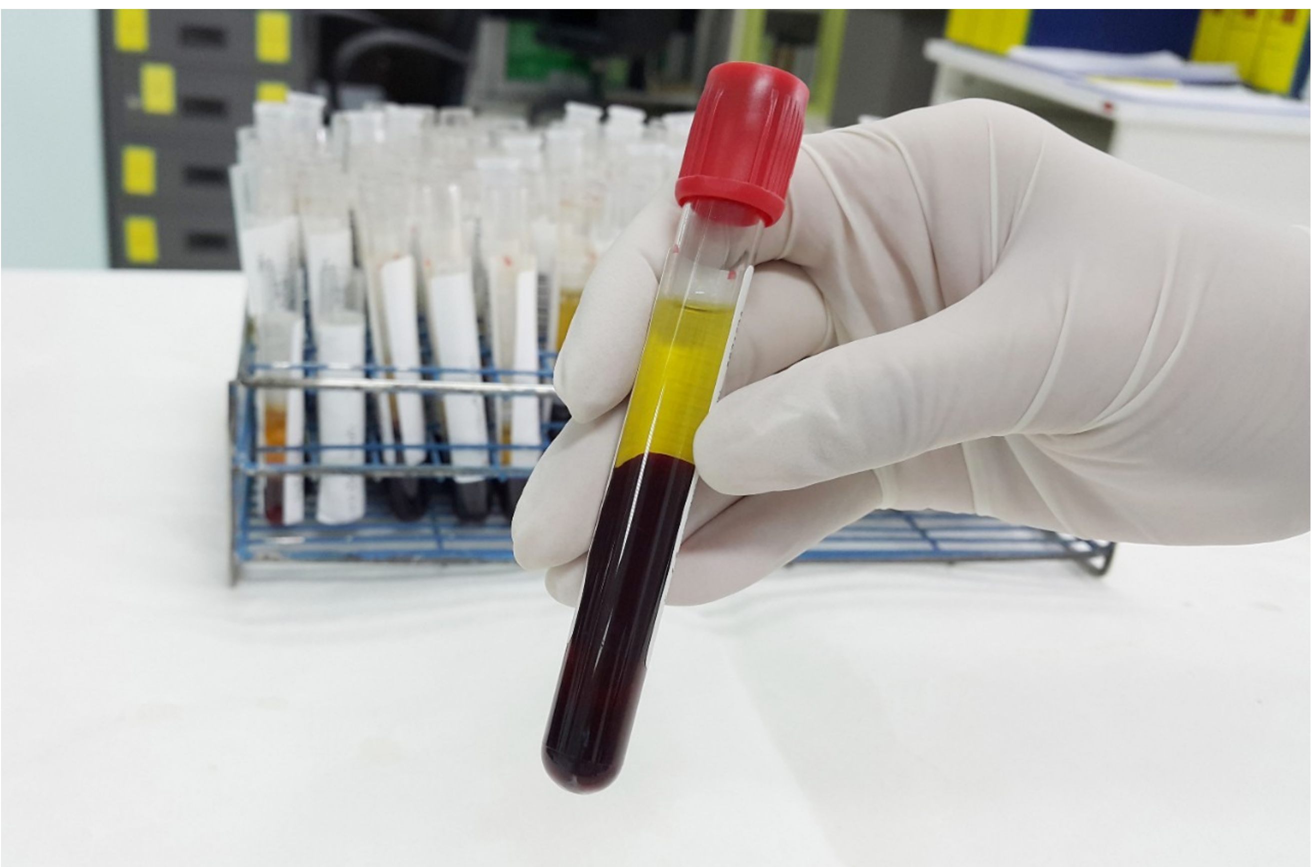


Abstract

Today's bioanalytical scientists face several challenges in their workflow, regardless of the regulated or non-regulated environment. Some of these challenges include:

- The ability to develop highly sensitive LC-MS methods for any molecule
- Ensuring high reproducibility and robustness while maintaining the shortest possible run time
- Addressing regulatory guidelines
- Ensuring high throughput value

This application note focuses on quantitation of clopidogrel by following the MRM of the active pro-drug with an LLOQ of less than 1 pg/mL in plasma.

Benefits

Waters Regulated Bioanalysis System Solution is capable of addressing sensitivity challenges in the world of regulated bioanalysis.

Introduction

Clopidogrel is a prodrug, which takes action on an adenosine diphosphate (ADP) receptor on platelet cell membranes. The drug specifically and irreversibly inhibits the P2Y12 subtype of an ADP receptor, which is important in aggregation of platelets and cross-linking by the protein fibrin.

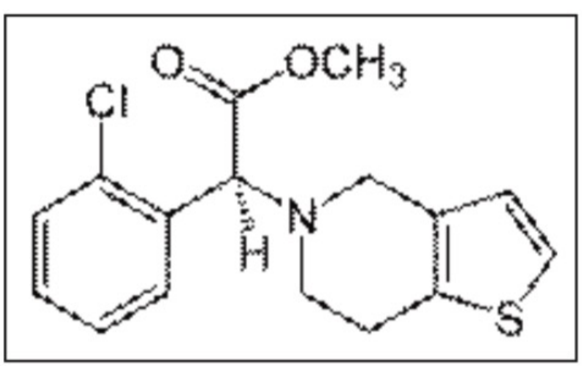


Figure 1. Molecular structure of clopidogrel.

Clopidogrel, chemical structure shown in Figure 1, consists of an asymmetrical carbon at C-7 resulting in two enantiomers, *R* and *S*. It has been reported that the S-enantiomer is the active compound1,[2] and the clopidogrel free base is susceptible to racemization and hydrolysis of methyl ester group.[2,3] Owing to the extensive and first-pass metabolism, clopidogrel and its active metabolite are not typically detected in plasma. The major circulating compound, which is also used for documenting the pharmacokinetic (PK) profile of clopidogrel, is its inactive carboxylic derivative. Such a PK profile is easily achieved by most HPLC and mass spectrometers due to the high abundance (~85%) of the inactive carboxylate metabolite of clopidogrel in human plasma.[4]

As mentioned above, the traditional detection of clopidogrel by its inactive carboxylate metabolite is obtained by an indirect method of documenting the PK profile. However, based on the characteristic low C_{max} exhibited by clopidogrel, it is necessary to estimate the drug in pg/mL concentration levels, which requires LC-MS instruments that are capable of achieving unforeseen sensitivity. In this application note, we report an LC-MS/MS method to determine and quantify clopidogrel at pg/mL or sub-pg/mL concentration levels and monitor the active pro-drug, clopidogrel, and not the inactive metabolite

This application note demonstrates the benefits of combining micro-elution and standard solid phase extraction methodology of Oasis Sample Extraction Products, the ACQUITY UPLC System, and the Xevo TQ-S Mass Spectrometer for the development of an LC-MS method to detect clopidogrel in sub-picogram quantity. In addition to addressing the challenge of achieving maximum sensitivity, the components of Waters Bioanalysis System Solution enables users to address several other challenges, including high selectivity and throughput.

The plasma samples were isolated using Oasis WAX 1 cc, 30 mg cartridges and micro-elution plates. A 400-µL aliquot of plasma was diluted with 10% formic acid and loaded onto the SPE cartridges previously conditioned with organic solvent and water. The plasma solution was then washed with acidified water followed by an organo-aqueous solution, then eluted in an organo-aqueous elution solvent. The eluted samples were directly injected onto the system. For micro-elution plates, the elution resulted in a higher concentration of the sample, and hence higher a signalto- noise (S/N) ratio compared to that obtained from the standard SPE. Atorvastatin was used as the internal standard (IS) for the estimation of clopidogrel.

Experimental

LC Conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC HSS C_{18} 1.8 µm, 2.1 x 50 mm
LC column:	99% aqueous buffer over 0.4 min followed by a 90% organic elution until 2 min; then change back to init conditions.
Column temp.:	40 °C
Flow rate:	0.400 mL/min
Injection volume:	5 µL

MS Conditions

MS system:	Xevo TQ-S
MS mode:	ESI positive
MRM transition:	322.1 → 212.0

Results and Discussion

Clopidogrel was eluted at 1.34 minutes, while the internal standard was eluted at 1.15 min with a peak width of 8 s at the base, as shown in Figure 2. The chromatographic method developed using the ACQUITY UPLC System and the ACQUITY UPLC HSS C_{18} Column provided excellent resolution for the clopidogrel analyte from the peaks of possible endogenous components in the extracted plasma samples. The signal-to-noise obtained for the LLOQ was 15 (average calculated from six replicate LLOQ injections, shown in Figure 3). The data shown in Figure 4.1 and Figure 4.2 exhibit the chromatogram obtained for the blank plasma and that of the clopidogrel analyte in LLOQ concentration of 0.5 pg/mL.

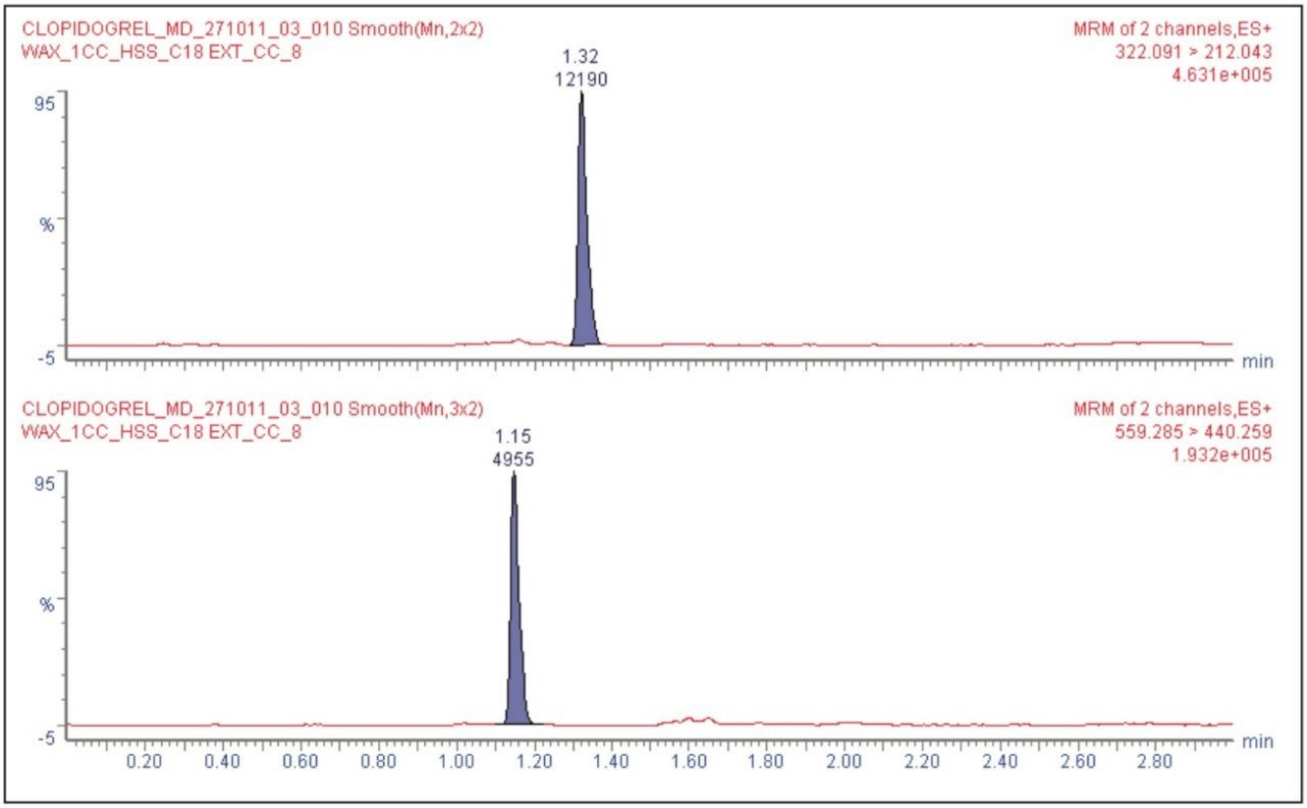
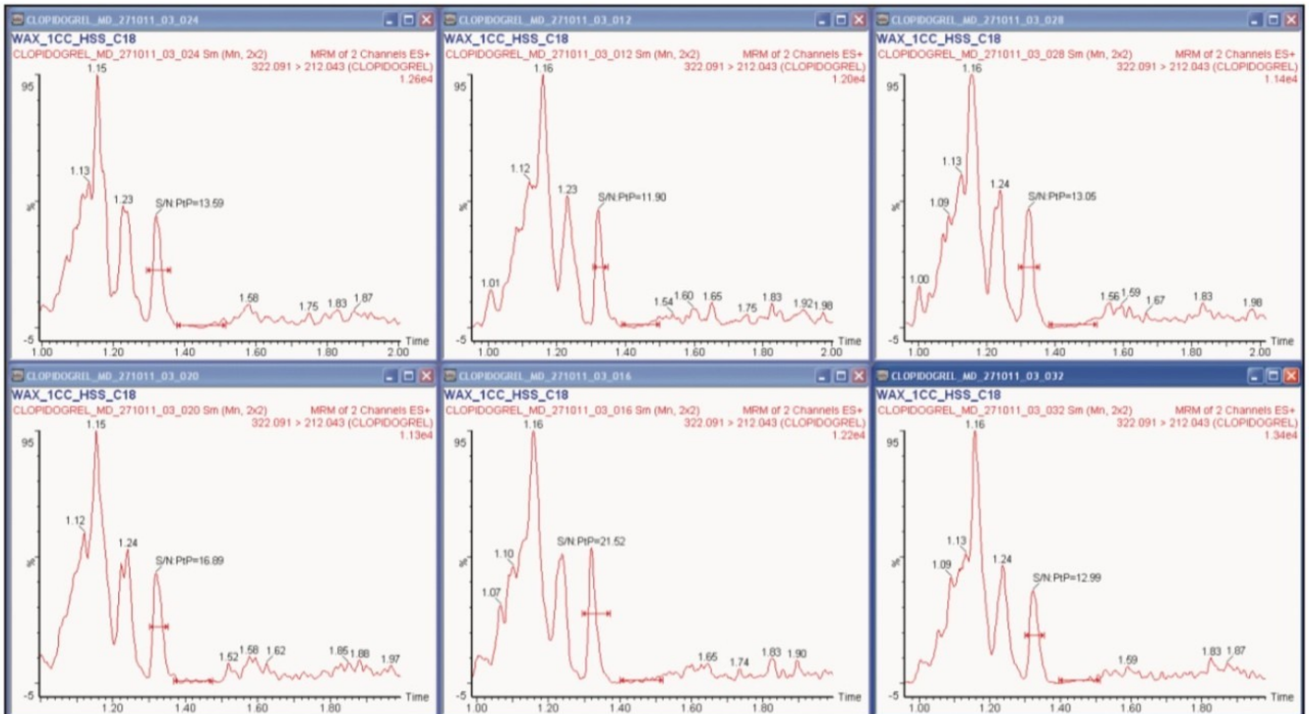


Figure 2. Elution pattern of clopidogrel and internal standard.



Signal-to-Noise Ratio of 6 replicates of LLOQ						
LLOQ1	LLOQ2	LLOQ3	LLOQ4	LLOQ5	LLOQ6	AVERAGE
13.59	11.90	13.05	16.89	21.52	12.99	14.99

Figure 3. Chromatogram of six batches of clopidogrel at the LLOQ concentration (0.5 pg/mL) with an average S/N ratio of ~15.

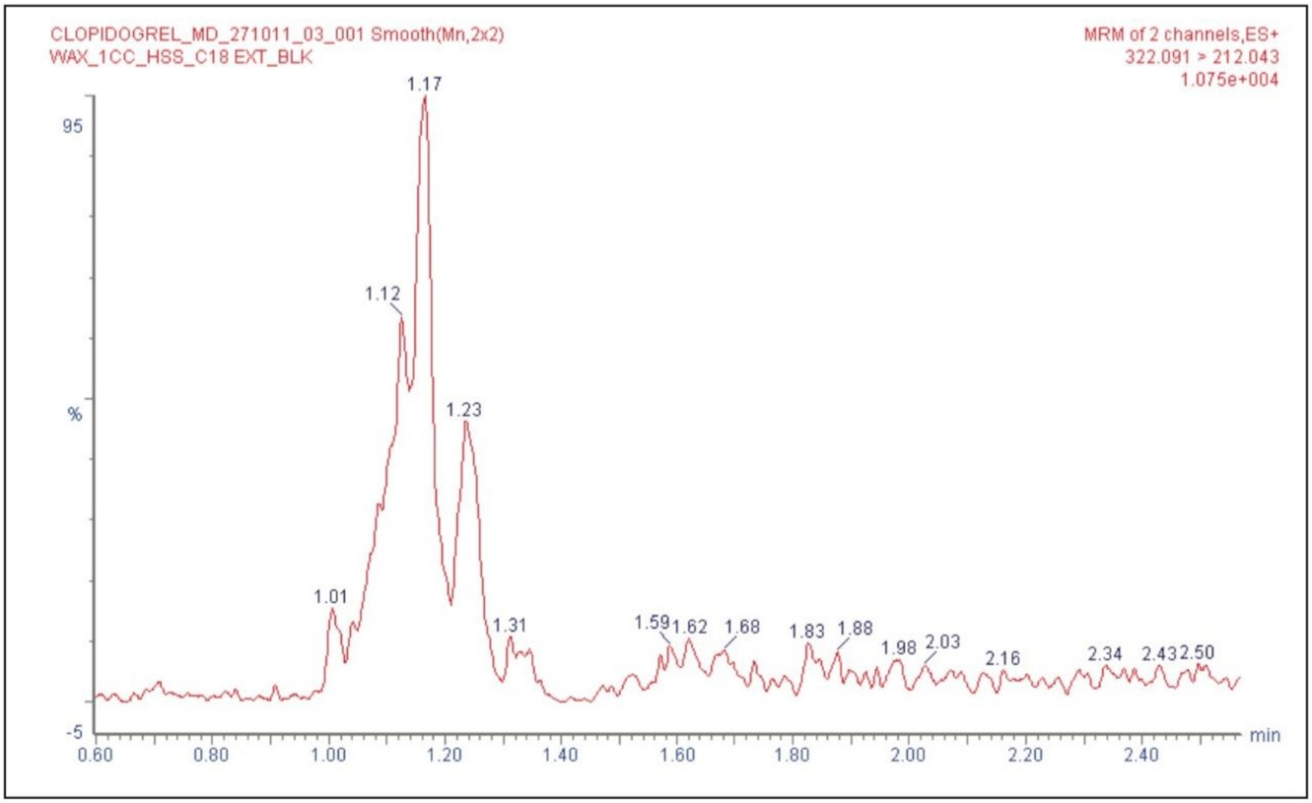


Figure 4.1 Chromatogram of the blank internal standard.

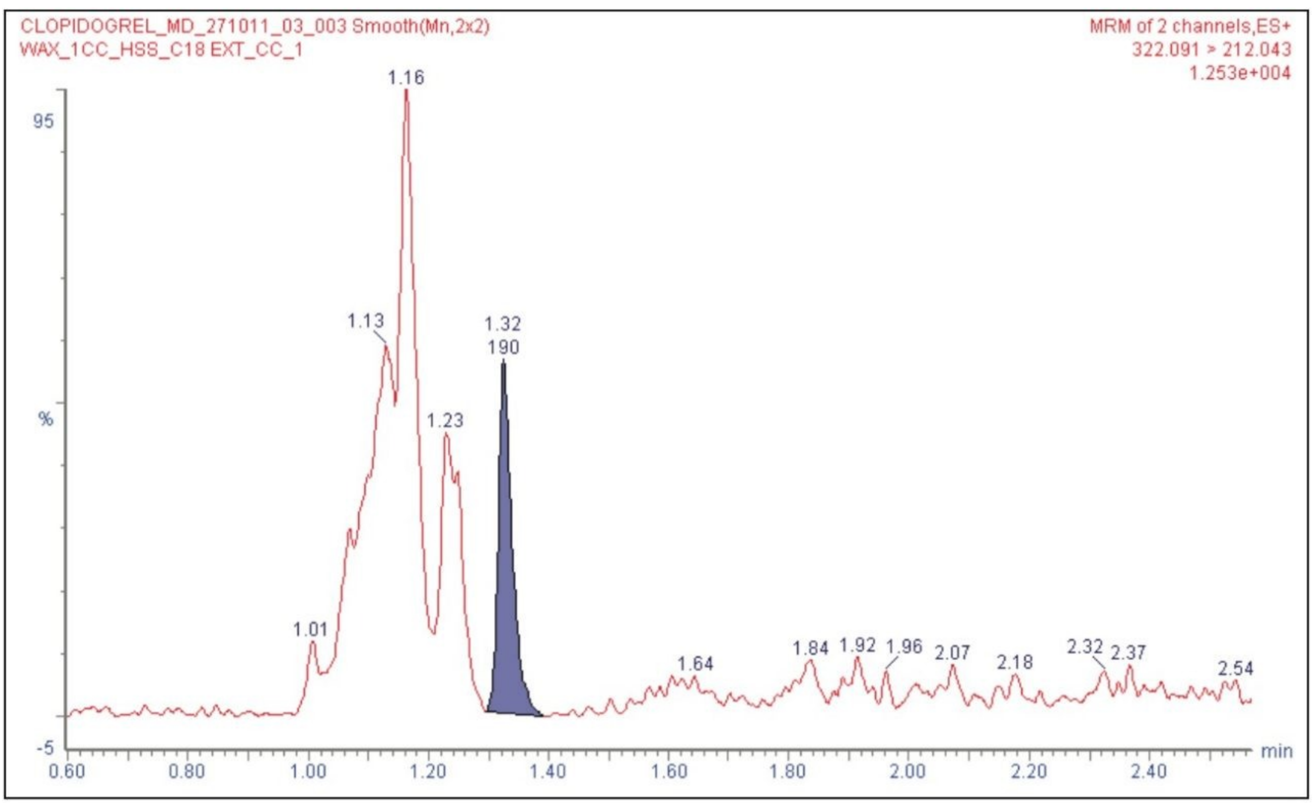


Figure 4.2. LLOQ concentration (0.5 pg/mL) clopidogrel.

As shown in Figures 4.1 and 4.2, clopidogrel eluted with a retention time that was well separated from the co-eluting peaks, arising from possible endogenous components of the plasma.

The assay in this report showed linear calibration over the range of 0.5 to 64.0 pg/mL with an r^2 value of 0.992, as shown in Figure 5. The back-calculated concentration of the standard was found to be within ± 8% of the nominal concentration, as shown in Table 1. This assay was performed with a three min injection-to-injection time. The nominal deviation observed for the data, shown in Table 1, and the short injection-to-injection time enables the user to address several challenges in the world of regulated bioanalysis, such as addressing regulatory concerns while ensuring a high-throughput.

Sample	Type	Nominal (pg/mL)	Analyte Area	ISTD Area	Area Ratio	Calculated (pg/mL)	Accuracy
EXT_BLK	Blank		4	27	0.1658		
EXT_BLK_IS	Blank		37	6152	0.0061		
EXT_CC_1	Standard	0.5	236	6255	0.0377	0.517	103.31
EXT_CC_2	Standard	1.0	371	6284	0.0591	0.957	95.65
EXT_CC_3	Standard	2.0	636	6193	0.1026	1.850	92.51
EXT_CC_4	Standard	4.0	1379	6294	0.2191	4.240	106.00
EXT_CC_5	Standard	8.0	2642	6555	0.4031	8.018	100.22
EXT_CC_6	Standard	16.0	4877	6422	0.7595	15.331	95.82
EXT_CC_7	Standard	32.0	10448	6194	1.6869	34.365	107.39
EXT_CC_8	Standard	64.0	18145	5848	3.1027	63.425	99.10

Table 1. Calibration data of clopidogrel over the range of 0.5 to 64.0 pg/mL.

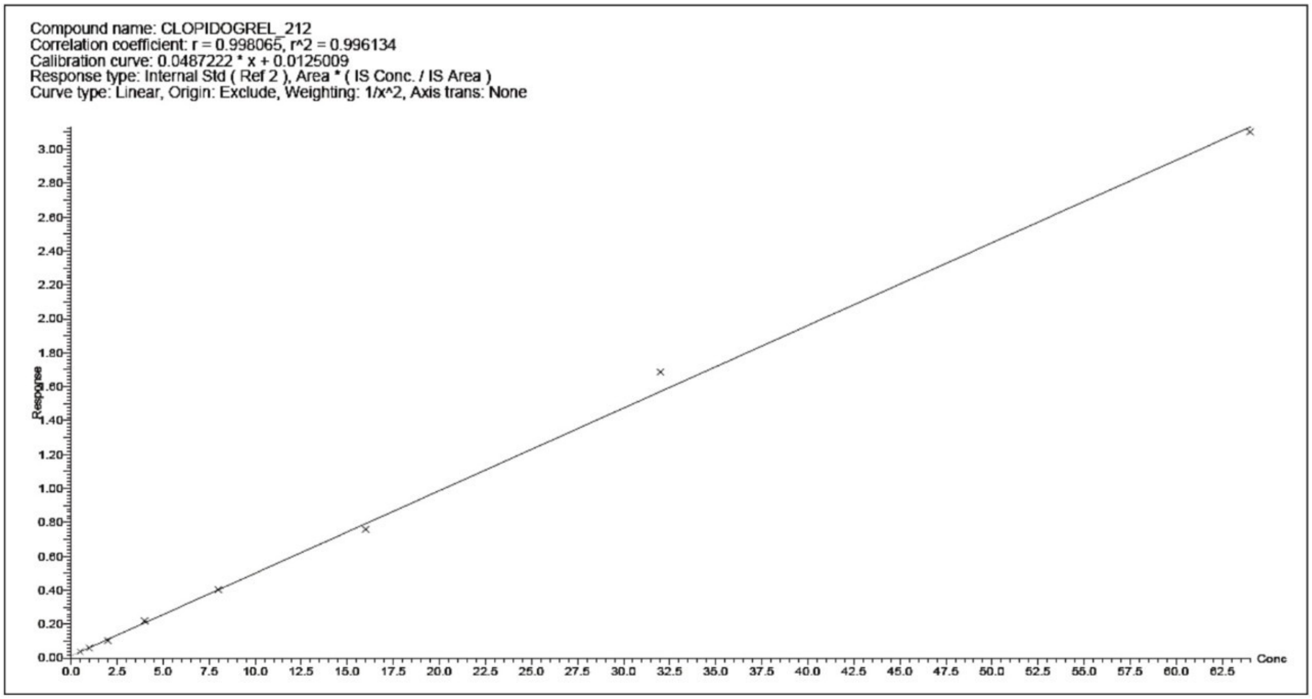


Figure 5. Calibration curve for clopidogrel.

Recovery of the analyte and IS was performed by comparison of extracted QC samples against six postextracted QC samples, and was found to be approximately 84% at LQC, MQC, and HQC levels for both analyte and IS, as shown in Figures 6.1, 6.2, and 6.3, and Table 2. The %CV for repeat batches were found to be within 10% of LLOQQC and varied between 1% and 3% for all QC levels.

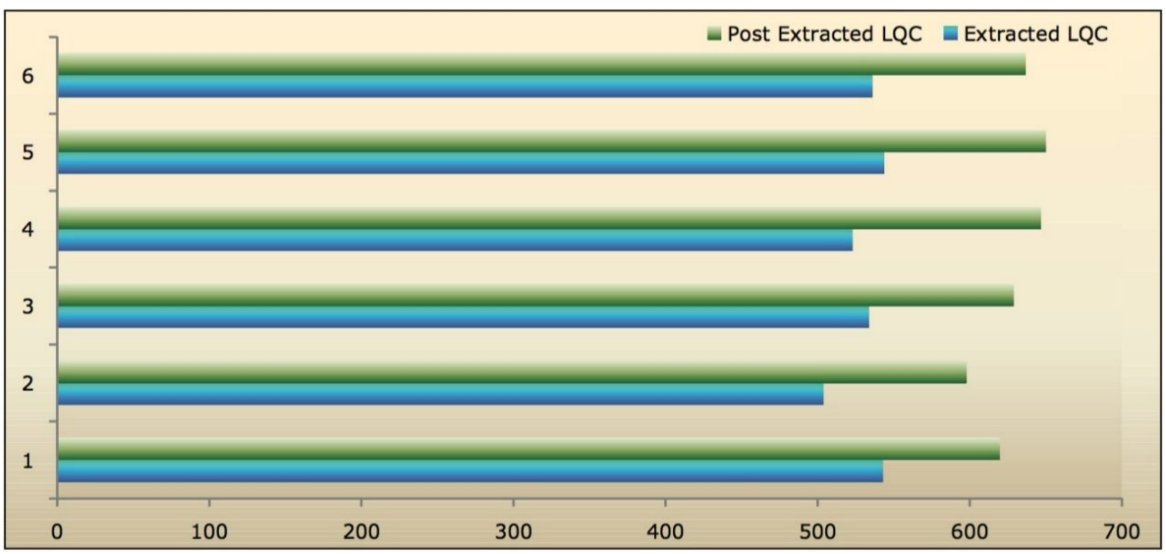


Figure 6.1. Analyte recovery (area under the curve) from six samples of clopidogrel at LQC, concentrations.

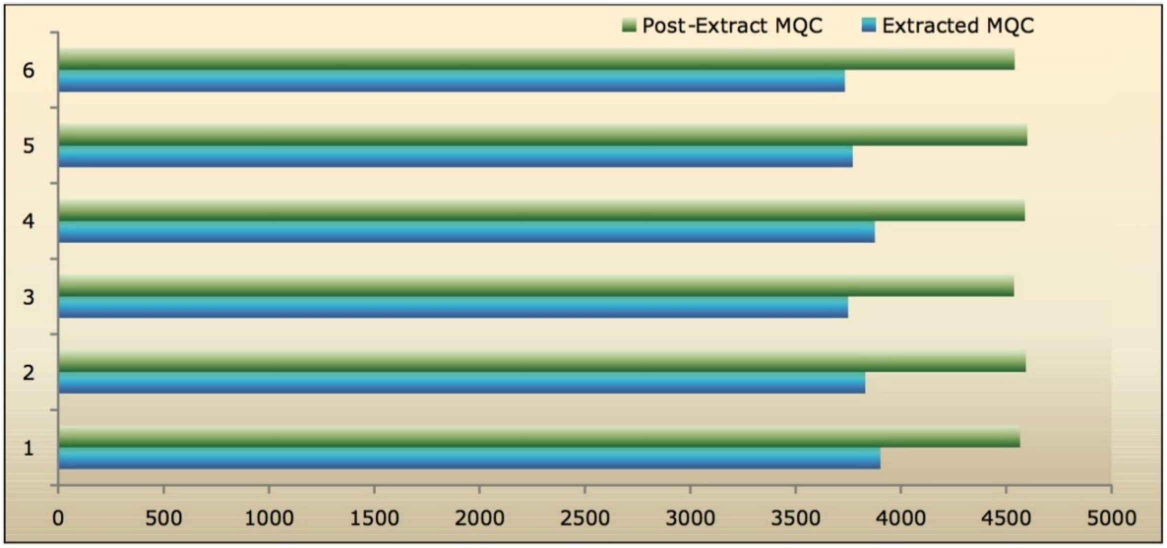


Figure 6.2. Analyte recovery (area under the curve) from six samples of clopidogrel at MQC, concentrations.

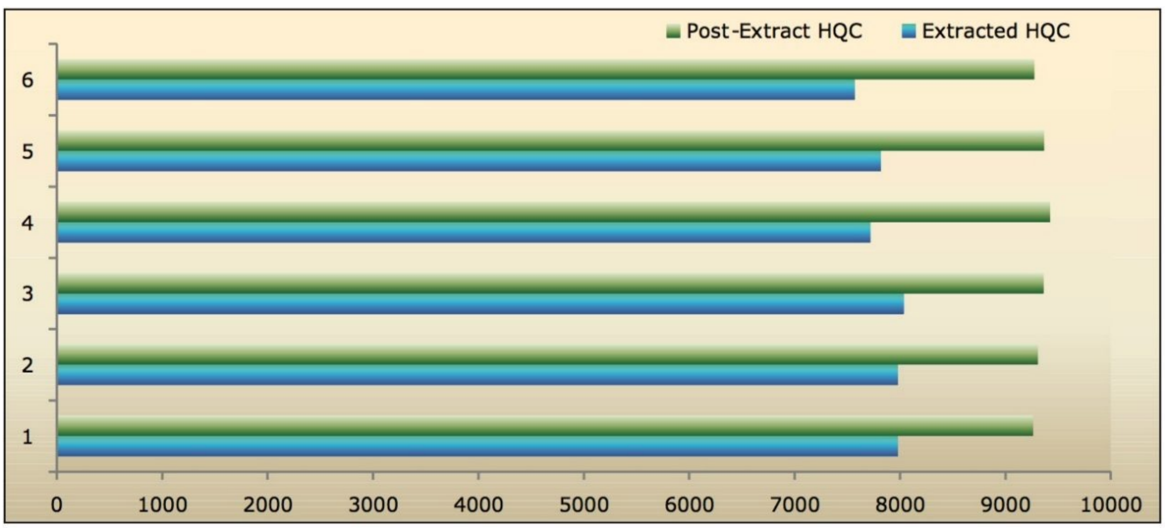


Figure 6.3. Analyte recovery (area under the curve) from six samples of clopidogrel at HQC, concentrations.

LQC	MQC	HQC
84.2	83.4	84.1
Mean Analyte Recovery (%) = 84		

Table 2. Mean analyte recovery (%) of clopidogrel at LQC, MQC, and HQC levels.

The data reported in Table 2 and Figures 6.1, 6.2, and 6.3 exhibit the remarkable consistency of the analyte recovery values for the six samples of clopidogrel for all three concentration levels (LQC, MQC, and HQC). In addition, as detailed in Table 2, the mean analyte recovery for the three concentration ranges was 84%. Such quality of data indicates the capability of Waters Regulated Bioanalysis System Solution for addressing robustness and consistency of the methods while addressing sensitivity challenges.

For a comparison of samples within the global batches, three separate batches were prepared with six samples in each batch for LLOQQC, LQC, MQC, and HQC concentration levels. The data showed excellent agreement between the six samples in all the three batches, as shown in Table 3. The mean accuracy obtained for all the samples was found to be > 94% for every concentration, as shown in Table 3.

P-A Batch-Global								
P-A-Batch-01	LLOQQC-1	0.512	LQC-1	1.925	MQC-1	16.167	HQC-1	32.426
	LLOQQC-2	0.430	LQC-2	2.005	MQC-2	15.345	HQC-2	31.716
	LLOQQC-3	0.432	LQC-3	1.808	MQC-3	15.468	HQC-3	30.170
	LLOQQC-4	0.448	LQC-4	1.765	MQC-4	15.867	HQC-4	32.701
	LLOQQC-5	0.429	LQC-5	1.887	MQC-5	15.293	HQC-5	33.019
	LLOQQC-6	0.404	LQC-6	2.081	MQC-6	15.083	HQC-6	32.167
P-A-Batch-02	LLOQQC-1	0.472	LQC-1	1.882	MQC-1	15.905	HQC-1	30.321
	LLOQQC-2	0.378	LQC-2	2.284	MQC-2	14.762	HQC-2	32.365
	LLOQQC-3	0.538	LQC-3	1.730	MQC-3	15.744	HQC-3	30.081
	LLOQQC-4	0.411	LQC-4	2.293	MQC-4	14.649	HQC-4	32.360
	LLOQQC-5	0.498	LQC-5	1.696	MQC-5	15.077	HQC-5	29.385
	LLOQQC-6	0.404	LQC-6	2.033	MQC-6	14.639	HQC-6	31.366
P-A-Batch-03	LLOQQC-1	0.548	LQC-1	1.942	MQC-1	15.318	HQC-1	33.095
	LLOQQC-2	0.456	LQC-2	2.021	MQC-2	14.951	HQC-2	33.180
	LLOQQC-3	0.447	LQC-3	1.946	MQC-3	15.498	HQC-3	32.360
	LLOQQC-4	0.483	LQC-4	1.903	MQC-4	15.501	HQC-4	31.402
	LLOQQC-5	0.391	LQC-5	2.020	MQC-5	15.156	HQC-5	32.719
	LLOQQC-6	0.526	LQC-6	1.878	MQC-6	15.364	HQC-6	30.763
P-A-Batch-04	LLOQQC-1	0.463	LQC-1	2.072	MQC-1	15.100	HQC-1	30.406
	LLOQQC-2	0.558	LQC-2	1.756	MQC-2	14.487	HQC-2	30.807
	LLOQQC-3	0.578	LQC-3	1.982	MQC-3	14.537	HQC-3	30.187
	LLOQQC-4	0.532	LQC-4	1.791	MQC-4	14.715	HQC-4	29.967
	LLOQQC-5	0.518	LQC-5	1.993	MQC-5	14.181	HQC-5	30.530
	LLOQQC-6	0.530	LQC-6	1.865	MQC-6	13.754	HQC-6	29.788
Mean		0.474		1.940		15.107		31.387
SD		0.0583		0.1526		0.5715		1.2106
%CV		12.29		7.87		3.78		3.86
Accuracy		94.88333		96.99583		94.41693		98.08346

Table 3. Comparison of the three separate batches each containing six clopidogrel samples at the LLOQQC, LQC, MQC, and HQC concentrations.

Conclusion

Clopidogrel is a thienopyridine derivative, which is used to prevent thrombosis after coronary artery stenting. The PK profile of clopidogrel is typically studied in an indirect method by quantification of an inactive carboxylate metabolite of clopidogrel. This method is primarily adopted because of the inability of most LC-MS instruments in achieving the desired sensitivity to monitor the direct clopidogrel drug and/or its active metabolite. In this study, the components of the Regulated Bioanalysis System Solution were successfully used to obtain highly sensitive LC-MS data with clopidogrel. The clopidogrel analyte was monitored instead of its inactive metabolite for detection and quantification. These results highlight the capabilities of Waters Regulated Bioanalysis System Solution in addressing sensitivity challenges in the world of regulated bioanalysis.

References

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