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A Validated Liquid-Liquid Extraction Method with Direct Injection of Hexane for Clopidogrel in Human Plasma Using UltraPerformance Convergence Chromatography (UPC^2) and Xevo TQ-S

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APPLICATION BENEFITS

Direct injection of bioanalytical liquid-liquid extracts (LLE) onto UPC$^{2®}$/MS instrumentation without the need for evaporation and reconstitution.

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ACQUITY UPC$^{2™}$ System

Xevo® TQ-S Mass Spectrometer

KEY WORDS

Convergence chromatography, UPC^2, tandem quadrupole MS, triple quadrupole MS, liquid-liquid extraction, LLE, clopidogrel

INTRODUCTION

Clopidogrel, shown in Figure 1, is a thienopyridine derivative antiplatelet pro-drug used in the prevention of artherosclerotic events. Following oral administration, the dosed compound undergoes hepatic metabolism to give 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]

Many bioanalytical methods utilize liquid-liquid extraction (LLE) by incorporating a non-polar solvent, such as hexane or methyl-tert-butyl ether. The widespread utilization of this choice of sample preparation is due to the ability of LLE to produce a much cleaner extract compared to protein precipitation techniques. In addition, LLE methods are relatively inexpensive when compared to other sample preparation methods.[2] However, the use of LLE requires dry-down and reconstitution steps into a more polar solvent that is readily compatible with typical starting conditions of revered-phase LC, which is the most common form of LC utilized in bioanalytical LC/MS/MS analysis.

This application note demonstrates the use of convergence chromatography in the development and validation of a highly sensitive UPC^2/MS/MS assay for the direct analysis of clopidogrel in human plasma from a hexane LLE preparation.

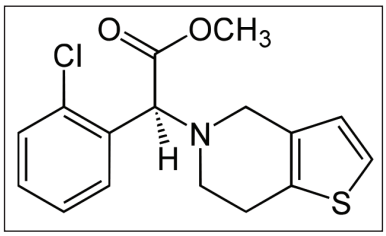

Figure 1. Structure of clopidogrel.

EXPERIMENTAL

Sample preparation

200 µL of human plasma was mixed with 20 µL of internal standard, then extracted with 600 µL of hexane. Samples were vortex mixed, centrifuged, and then the supernatant was transferred to a total recovery LC vial.

A 10-µL sample was injected onto an ACQUITY UPC^2 System. Chromatography was performed on an ACQUITY UPC^2 BEH 3.0 x 100 mm, 1.7 µm Column maintained at 40 °C. The column was operated under linear gradient conditions, starting at 98:2 CO_2/0.1% formic acid in acetonitrile to 70:30 in 2 min at a flow rate of 1.4 mL/min.

The column effluent was monitored using a Xevo TQ-S 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 d4 internal standard.

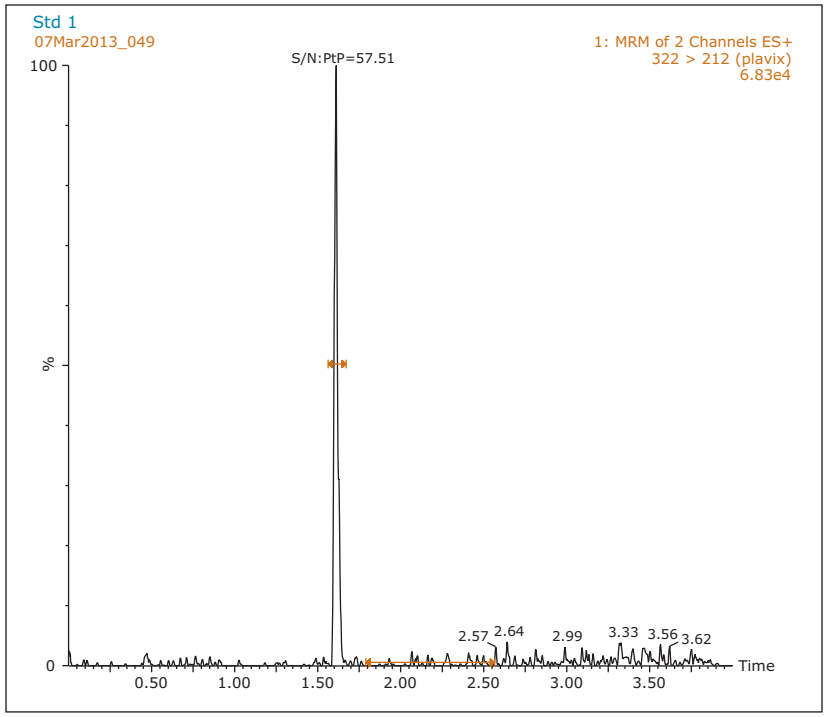

Figure 2. Signal to noise for the lowest standard prepared at 25.0 pg/mL.

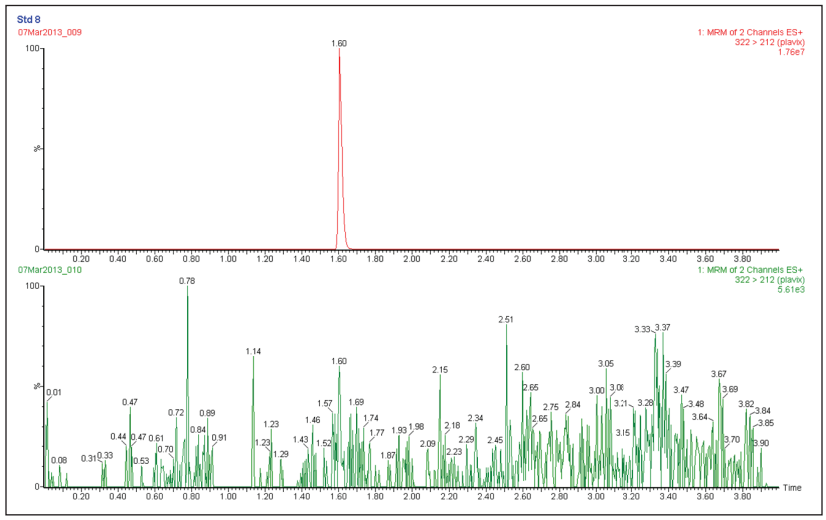

Figure 3. UPC^2/MS/MS chromatogram of extracted blank prepared at the upper LOQ, 5000 ng/mL.

RESULTS AND DISCUSSION

Clopidogrel eluted with a retention time of 1.6 minutes, as shown in Figure 2. Data show that the peak produced by the chromatography system was symmetrical and narrow with a width measured at peak base of three seconds. There is also very little background noise present using this method, facilitating a signal-to-noise value of approximately 60:1 for the lowest standard, as shown in Figure 2.

The data in Figure 3 illustrates the injection of an extracted plasma blank injection immediately following the analysis of a 5000 pg/mL standard. There is no carryover in the blank chromatogram, critically important for any bioanalytical method.

The assay was validated with separate accuracy and precision batches on three consecutive days ranging from 25 to 5000 pg/mL. A typical calibration obtained for the assay is shown in Figure 4, whereby the correlation coefficient ranged between 0.996 and 0.999 using a 1/x weighting linear regression. The intra-day precision and accuracy validation data are displayed in Tables 1 through 3. The validation data show that the coefficient of variation ranged from 1.8% to 6.3% for the 25.0 pg/mL LLOQ, with a bias between -13.2% and 10.2%. The high QC (3750 pg/mL) coefficient of variation ranged from 2.9% to 7.8% with a bias between -7.8% and -0.2%. The inter-day precision and accuracy data are displayed in Table 4. The coefficient of variation was determined to be 11.1% for the 25.0 pg/mL LLOQ with a bias of -2.0%. For the high QC (3750 pg/mL), the coefficient of variation was determined to be 6.2% with a bias of 2.0%.

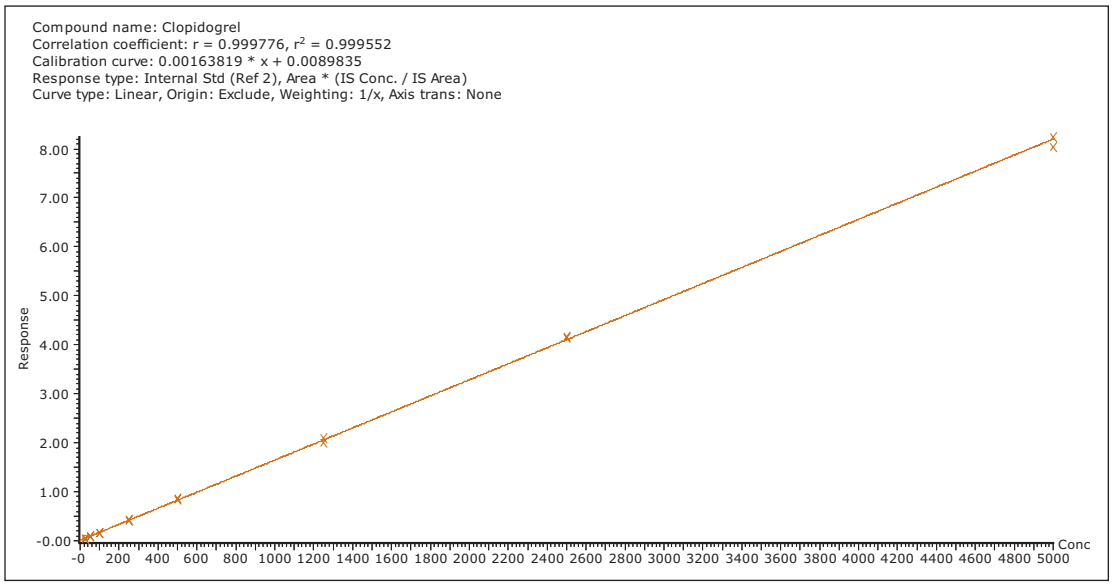

Figure 4. Representative calibration line for the UPC²/MS/MS quantification of clopidogrel in plasma.

	QC LLOQ 25.0pg/mL	QC Low 75.0pg/mL	QC Mid 350pg/mL	QC High 3750pg/mL
	23.6	71.6	355	3384
	23.4	76.5	358	3422
	25.0	67.2	363	3299
	25.9	72.8	349	3558
	23.3	74.0	346	3629
Mean	24.2	72.4	354	3458
St Dev	1.15	3.44	6.7	133
%CV	4.8	4.7	1.9	3.9
%Bias	-3.0	-3.4	-5.5	-7.8

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

	QC LLOQ 25.0pg/mL	QC Low 75.0pg/mL	QC Mid 350pg/mL	QC High 3750pg/mL
	21.4	74.6	404	3464
	22.3	80.4	379	3577
	21.8	68.9	390	3395
	21.7	74.2	370	3647
	21.3	69.9	361	3459
Mean	21.7	73.6	381	3508
St Dev	0.39	4.57	16.5	101
%CV	1.8	6.2	4.3	2.9
%Bias	-13.2	-1.9	1.6	-6.4

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

	QC LLOQ 25.0pg/mL	QC Low 75.0pg/mL	QC Mid 350pg/mL	QC High 3750pg/mL
	28.0	69.8	358	3684
	27.1	74.3	340	3257
	27.7	68.3	346	3940
	29.9	75.7	395	3967
	25.1	67.7	347	3857
Mean	27.6	71.2	357	3741
St Dev	1.73	3.62	21.9	292
%CV	6.3	5.1	6.1	7.8
%Bias	10.2	-5.1	-4.8	-0.2

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

	QC LLOQ 25.0pg/mL	QC Low 75.0pg/mL	QC Mid 350pg/mL	QC High 3750pg/mL
	23.6	71.6	355	3384
	23.4	76.5	358	3422
	25.0	67.2	363	3299
	25.9	72.8	349	3558
	23.3	74.0	346	3629
	21.4	74.6	404	3464
	22.3	80.4	379	3577
	21.8	68.9	390	3395
	21.7	74.2	370	3647
	21.3	69.9	361	3459
	28.0	69.8	358	3684
	27.1	74.3	340	3257
	27.7	68.3	346	3940
	29.9	75.7	395	3967
	25.1	67.7	347	3857
Mean	24.5	72.4	364	3569
St Dev	2.73	3.76	19.5	221
%CV	11.1	5.2	5.4	6.2
%Bias	-2.0	-3.5	4.0	2.0

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

CONCLUSIONS

- A high sensitivity method for the direct injection of an LLE extract has been developed for the analysis of clopidogrel in human plasma utilizing UPC^2/MS/MS technology.
- The assay showed excellent intra- and inter-day accuracy and precision in a three-day validation study.
- The level of quantification was determined to be 25.0 pg/mL with a signal-to-noise ratio of approximately 60:1 with the %CV and bias both less than +/- 15%.
- 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

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