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A High Sensitivity UPLC/MS/MS Method for the Analysis of Clopidogrel and Clopidogrel Carboxylic Acid Metabolite in Human K_2EDTA Plasma

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APPLICATION BENEFITS

A high-sensitivity method for the analysis of clopidogrel and its carboxylic metabolite was developed using solid-phase extraction along with UPLC/MS/MS that achieved an LLOQ of 1 pg/mL in human plasma.

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KEY WORDS

Clopidogrel, clopidogrel carboxylic acid metabolite

INTRODUCTION

Clopidogrel (trade name Plavix, Figure 1), is a thienopyridine derivative antiplatelet prodrug used in the prevention of artherosclerotic events. It is dosed in an inactive form, and requires a hepatic biotransformation to yield the active thiol-metabolite which binds to cell receptor P2Y12, irreversibly inhibiting the platelet activation process.[1]

In addition to the active metabolite, an inactive carboxylic acid metabolite is also formed. This acid metabolite accounts for the majority of circulating clopidogrel related material with very low levels of the active metabolite and unchanged prodrug being present.[2,3] Due to the reactivity of the thiol metabolite, coupled with the low levels of the unchanged prodrug, most quantitative studies are based on the circulating levels of the inactive metabolite.

The ability to detect the low levels of unchanged prodrug will provide more accurate data on the pharmacokinetics of clopidogrel, allowing improved evaluation of the bioavailability of new formulations.

Most published methods for the analysis of clopidogrel/metabolite employ a liquid-liquid extraction (LLE) technique, often times requiring a double LLE prior to LC/MS.[3,4] This method is both time consuming and tedious and often requires large volumes of harmful chemicals, such as hexane and diethyl ether.

To address this issue the use of solid-phase µElution technology was investigated, with the aim to increase throughput while decreasing solvent consumption. A typical LLE will consume anywhere from 2 to 8 mL of organic solvent per sample, in contrast to µElution methods that require less than 0.5 mL of organic solvent. The use of µElution plates also allows the sample to be concentrated during the extraction process which facilitates lower detection limits.

In this application note, we present a high-sensitivity method for the analysis of clopidogrel and clopidogrel carboxylic acid metabolite from human plasma using UPLC/MS/MS.

EXPERIMENTAL

UPLC conditions

LC system:	Waters ACQUITY UPLC® System
LC column:	Waters ACQUITY UPLC BEH C_{18} 1.7-µm, 1.0 x 50 mm
Flow rate:	140 µL/min
Column temp.:	45 °C
Mobile phase A:	0.1% Formic acid
Mobile phase B:	Acetonitrile
Gradient:	10% B hold for 0.5 min 10 to 90% B from 0.5 to 3 min

MS conditions

MS System:	Waters Xevo® TQ-S equipped with a low flow probe for use with 1.0-mm I.D. columns
MS/MS Parameters:	
	Transitions: Clopidogrel 322.1 > 212.0
	d4-clopidogrel 326.1 > 216.1
	Clopidogrel metabolite 308.1 > 198.1
	d4-clopidogrel metabolite 312.1 > 202.1
Ionization:	Positive ion ESI
Capillary voltage:	0.5 kV
Collision energies:	16 V
Cone voltage:	35 V

Data integration and calculation software

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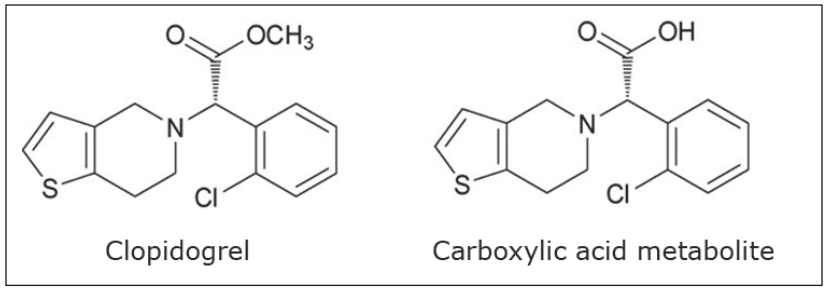

Figure 1. Chemical structure of clopidogrel and carboxylic metabolite.

SAMPLE PREPARATION

Solid phase extraction

Plasma samples were extracted by diluting 350 µL of plasma sample, containing 10 µL of internal standard solution at a concentration of 250 pg/mL, with 350 µL of aqueous 2% formic acid. Samples were added to an Oasis® HLB µElution plate, after pre-conditioning the plate with methanol (200 µL) followed by water (200 µL). Samples were drawn through under vacuum, and then washed with 2% formic acid (200 µL) and 5% methanol/water (200 µL). The sample was eluted with 2 x 25 µL of methanol, and then diluted with an equal volume of water prior to injection. Samples were prepared at the following concentrations: 1.00, 2.50, 5.00, 10.0, 25.0, 50.0, 100, 250, and 500 pg/mL.

RESULTS AND DISCUSSION

The resulting chromatograms obtained for standards clopidogrel and clopidogrel carboxylic acid, as well as the internal standards d_4-clopidgrel and d_4-clopidogrel carboxylic acid are shown in Figure 2.

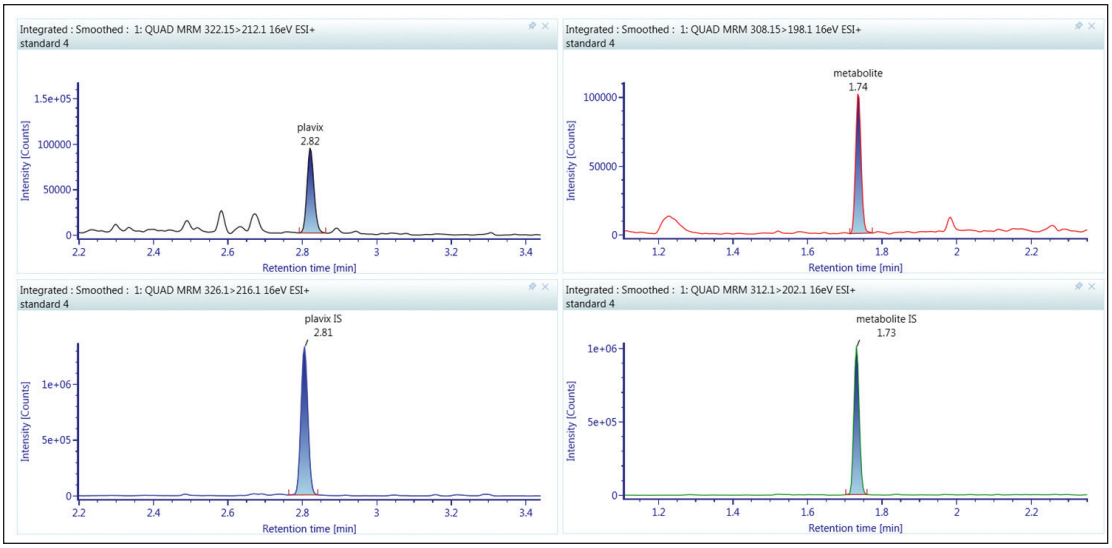

Figure 2. Example chromatograms of clopidogrel (top left), deuterated clopidogrel (bottom left), carboxylic acid metabolite (top right) and deuterated carboxylic acid metabolite (bottom right).

The metabolite and deuterated internal standard elute at 1.7 min, while clopidogrel and its internal standard elute at 2.8 min. The calibration curve was linear over the range of 1 to 500 pg/mL, with no carryover present (Figures 3 to 5).

The use of µElution technology along with a 1.0-mm I.D. column gave a lower limit of quantification (LLOQ) of 1 pg/mL for both clopidogrel and its metabolite. The use of microbore chromatography is known to give increases in sensitivity of up to four-fold over that obtained with a standard 2.1-mm column. Clopidogrel showed a signal-to-noise value of 7:1 while the metabolite showed signal-to-noise value of 5:1 (Figure 6). Quality control (QC) samples were injected in replicates of five at four different levels spanning the range of the calibration curve. All QCs met acceptance criteria of accuracy/precision ± 20% for the LLOQ and accuracy precision ± 15% for all QC levels (Table 1).

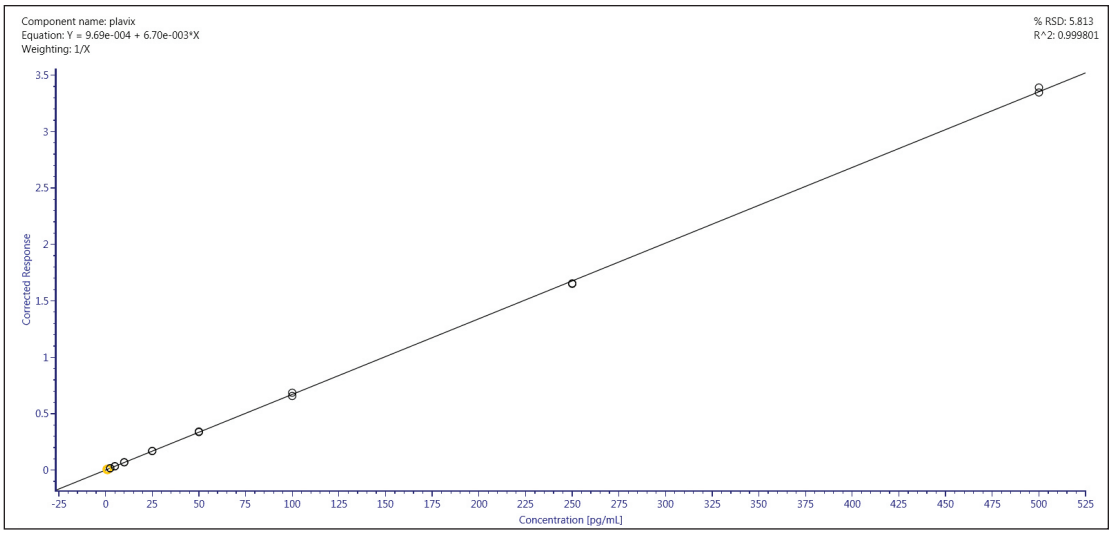

Figure 3. Calibration line for clopidogrel from 1 to 500 pg/mL.

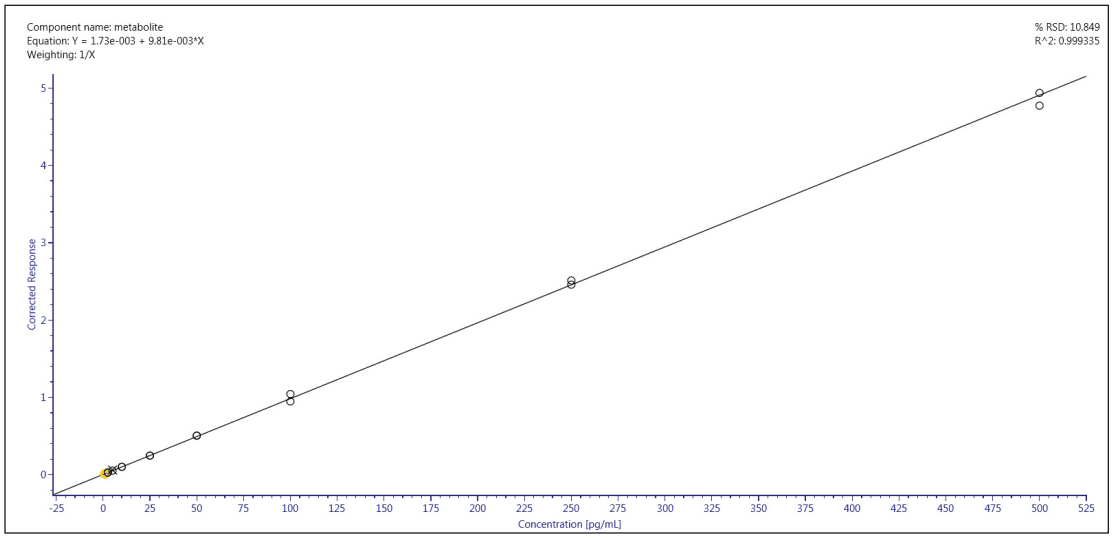

Figure 4. Calibration line for clopidogrel carboxylic acid metabolite from 1 to 500 pg/mL.

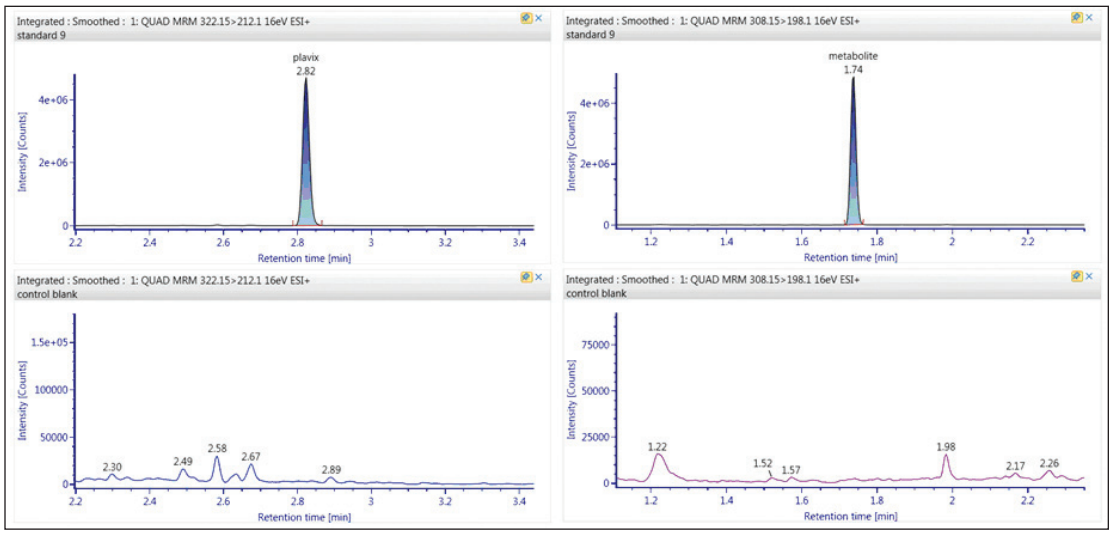

Figure 5. No detectable carryover was observed for both clopidogrel (ULOQ top left, blank bottom left) and the carboxylic acid metabolite (ULOQ top right, blank bottom right).

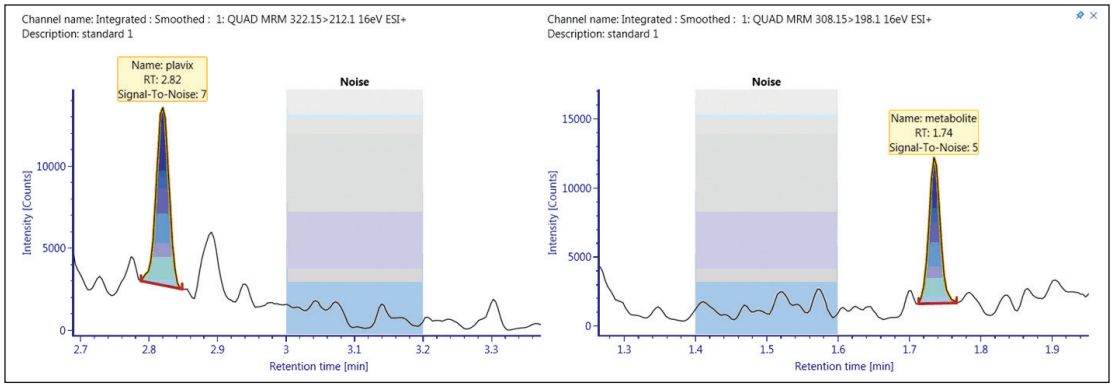

Figure 6. Signal-to-noise values for clopidogrel and carboxylic acid metabolite.

	Plavix QC Statistics					Metabolite QC Statistics			
	QC LLOQ	QC LOW	QC MID	QC HIGH		QC LLOQ	QC LOW	QC MID	QC HIGH
	1.00pg/mL	3.00pg/mL	30.0pg/mL	200pg/mL		1.00pg/mL	3.00pg/mL	30.0pg/mL	200pg/mL
	1.154	3.11	29.0	195		0.937	3.05	29.5	193
	1.29	3.09	30.9	203		0.973	3.48	29.9	199
	0.99	3.07	29.8	199		0.977	3.23	30.9	190
	1.036	3.04	30.0	204		0.907	2.94	30.6	198
	1.17	3.18	29.3	200		1.04	2.88	31.3	201
Mean	1.13	3.10	29.8	200	Mean	0.966	3.11	30.4	196
St Dec	0.118	0.0516	0.725	3.36	St Dec	0.0488	0.244	0.720	4.57
%CV	10.5	1.7	2.4	1.7	%CV	5.1	7.8	2.4	2.3
%Bias	12.8	3.2	-0.7	0.0	%Bias	-3.4	3.8	1.4	-1.9

Table 1. QC statistical data for QC levels at LLOQ, Low, Mid, and High values.

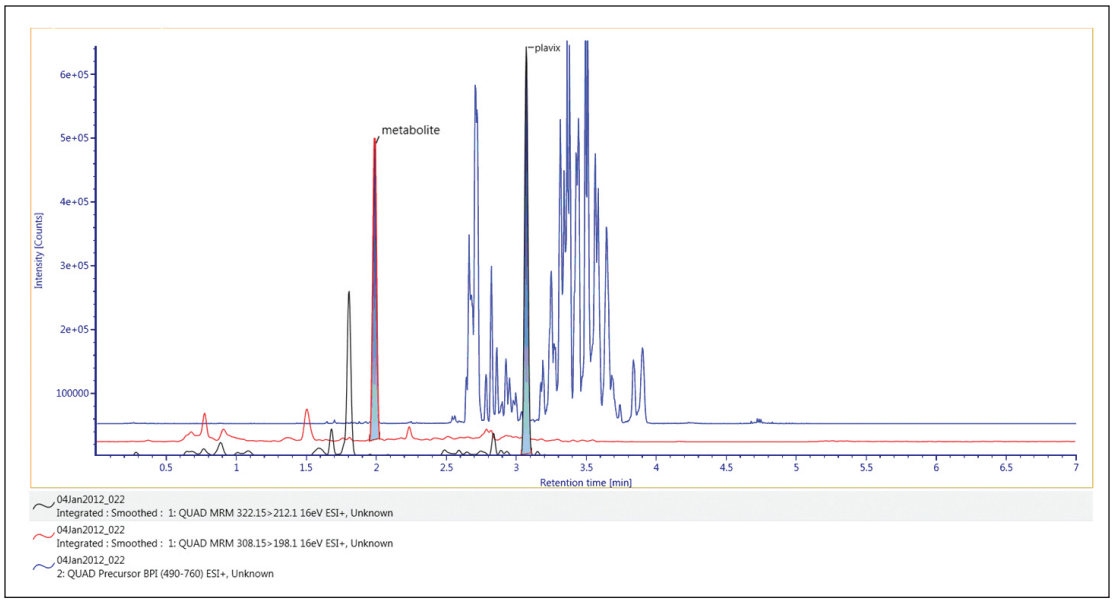

Figure 7. Chromatographic resolution of clopidogrel and carboxylic metabolite from choline-containing phospholipids.

Another essential part of method reliability and robustness is the ability to separate the analyte(s) of interest from any background interferences. The most common interferences encountered in bioanalysis are phospholipids, which can be especially problematic for late eluting compounds, such as clopidogrel. To determine if phospholipids would coelute with the drug and metabolite, the generic 184 >184 transition was monitored (Figure 7). As can be seen from the chromatogram, clopidogrel elutes in between two major regions of interferences while the metabolite elutes over a minute before any of the phospholipids.

CONCLUSIONS

- A high-sensitivity method for the analysis of clopidogrel and its carboxylic metabolite was developed with an LLOQ of 1 pg/mL
- Quality Control (QC) samples meet required acceptance criteria put forth by the U.S. FDA
- Both clopidogrel and the carboxylic acid metabolite were successfully separated from possible coeluting phospholipids
- There was no detectable carryover present

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