

## Application Data Sheet

No.04

### COVID-19 Stable isotope-labeled

LC-MS/MS Internal Standards references

## Method for multiple COVID-19 drugs analysis by LC-MS/MS

### Introduction

The COVID-19 pandemic accelerated the use of repurposed drugs to treat patients worldwide. The lack of knowledge about concentration and pharmacokinetics is an issue to propose the right dose. We developed and present here a LC-MS/MS method to monitor these drugs (Favipiravir, GS 441524, Remdesivir, Hydroxychloroquine, Desethylchloroquine, Chloroquine, Azithromycin, Ritonavir, Lopinavir) used in more than 50% of the clinical trials performed worldwide on COVID-19 patients.

### Methods

#### HPLC Conditions

Analytical column : C18 2.1x50 mm, 5 µm  
 Mobile phase A : H<sub>2</sub>O + 0.1% Formic acid  
 Mobile phase B : MeOH + 0.1% Formic acid  
 Flow rate : 0.5 mL/min  
 Oven temperature : 30 °C

#### MS Conditions

Ionization : ESI Positive  
 DL temp. : 200 °C  
 Heat Block temp. : 300 °C  
 Interface temp. : 250 °C  
 Nebulizer gas flow : 2 L/min  
 Drying gas flow : 10 L/min  
 Heating gas flow : 10 L/min

#### Sample preparation

This method includes 9 analytes (Favipiravir, GS 441524, Remdesivir, Hydroxychloroquine, Desethylchloroquine, Chloroquine, Azithromycin, Ritonavir, Lopinavir) and their corresponding Stable Isotope Labeled Standards.

Samples are prepared as follows:

- 1) Place 50 µL of plasma sample in 1.5 mL microtube
- 2) Add 200 µL of MeOH containing Internal standard
- 3) Vortex 1 min
- 4) Centrifuge 7 min at 15 000 g
- 5) Dilute the supernatant with mobile phase A: introduce 100 µL of mobile phase A in a vial with insert and add 50 µL of supernatant (dilution factor before analysis can be increased)

Injection volume: 1µL (depending on mass spectrometer sensitivity)

The treated samples were separated by analytical column at 30° C within 6 min. Gradient program is described in table 1. Detection of equipment is Shimadzu Nexera X2 LCMS-8050 with electrospray ionization in positive ion mode. Parameters for multiple reaction monitoring (MRM) are presented in table 2.

Table 1: Gradient program

Time (min)	event	%
0.00	Pump B conc.	1
3.00	Pump B conc.	99
4.00	Pump B conc.	99
4.50	Pump B conc.	1
6.00	Stop	

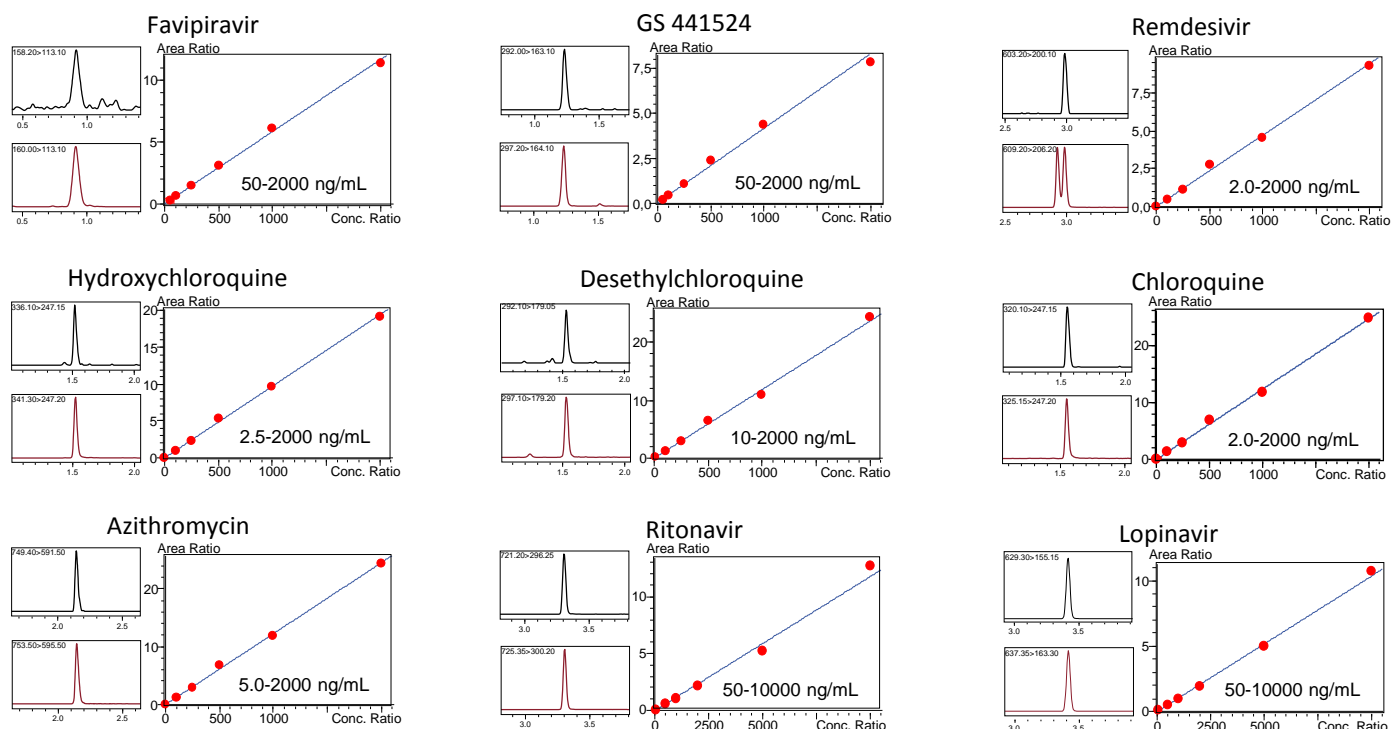
Table 2: MRM parameters

Molecules	Ref.	Transitions MRM (1)	Transitions MRM (2)
Favipiravir	C8720	158.20>113.10	158.20>85.10
GS 441524	C8847	292.00>163.10	292.00>147.10
Remdesivir	C8799	603.20>200.10	603.20>402.20
Hydroxychloroquine	C4600	336.10>247.15	336.10>179.10
Desethylchloroquine	C2331	292.10>179.05	292.10>114.20
Chloroquine	C1741	320.10>247.15	320.10>142.20
Azithromycin	C1746	749.40>591.50	749.40>158.20
Ritonavir	C2792	721.20>296.25	721.20>228.25
Lopinavir	C2745	629.30>155.15	629.30>447.30

Molecules	Ref.	Transitions MRM (1)	Transitions MRM (2)
[ <sup>13</sup> C, <sup>15</sup> N]-Favipiravir	C8853	160.00>113.10	160.00>85.10
[ <sup>13</sup> C <sub>6</sub> ]-GS 441524	C8855	297.20>164.10	297.20>148.10
[U-Ring- <sup>13</sup> C <sub>6</sub> ]-Remdesivir	C8854	609.20>206.20	609.20>408.35
[ <sup>2</sup> H <sub>5</sub> ]-Hydroxychloroquine	C6422	341.30>247.20	341.30>179.20
[ <sup>2</sup> H <sub>5</sub> ]-Desethylchloroquine	C2453	297.10>179.20	297.10>119.20
[ <sup>2</sup> H <sub>5</sub> ]-Chloroquine	C5023	325.15>247.20	325.15>147.25
[ <sup>13</sup> C, <sup>2</sup> H <sub>3</sub> ]-Azithromycin	C1768	753.50>595.50	753.50>158.25
[ <sup>13</sup> C, <sup>2</sup> H <sub>3</sub> ]-Ritonavir	C2963	725.35>300.20	725.35>272.20
[ <sup>2</sup> H <sub>8</sub> ]-Lopinavir	C4693	637.35>163.30	637.35>447.35

## Results

We carried out concurrent analysis over a concentration range up to 2000 ng/mL for 7 molecules and up to 10000 ng/mL for Ritonavir and Lopinavir. The calibration curves that were generated had linear regression values of  $r^2 > 0.99$  for each curve. CV% values of concentration were within acceptable analytical ranges (<15%) .



Black peak: unlabeled compound

Red peak: labeled compound (for Remdesivir, labeled compound is a mixture of diastereoisomers).

	Favipiravir			GS 441524			Remdesivir		
Concentrations (ng/mL)	50	100	1000	50	100	1000	25	100	1000
Average (ng/mL)	49.8	99.8	1092.5	47.6	99.6	1035.2	23.1	97.8	988.9
CV (%)	4.4	8.6	5.9	10.6	6.9	4.6	4.1	3.7	1.9
Deviation (%)	-0.5	-0.2	9.3	-4.9	-0.4	3.5	-7.5	-2.2	-1.1

	Hydroxychloroquine			Desethylchloroquine			Chloroquine		
Concentrations (ng/mL)	25	100	1000	50	100	1000	25	100	1000
Average (ng/mL)	24.4	96.6	1003.0	47.6	95.2	949.2	24.2	95.6	963.2
CV (%)	3.6	3.0	2.4	4.4	2.2	5.6	3.3	3.0	1.8
Deviation (%)	-2.5	-3.4	0.3	-4.7	-4.8	-5.1	-3.2	-4.4	-3.7

	Azithromycin			Ritonavir			Lopinavir		
Concentrations (ng/mL)	25	100	1000	100	500	1000	100	500	1000
Average (ng/mL)	24.9	97.2	962.2	98.3	498.6	891.3	92.1	503.7	917.2
CV (%)	6.6	2.5	2.0	3.2	2.0	3.1	2.0	0.8	1.3
Deviation (%)	-0.6	-2.8	-3.8	-1.7	-0.3	-10.9	-7.9	0.7	-8.3

Figure 1. Calibration curves, MRM Chromatograms (at Calibrator Level 1) and summary of repeatability

N=6