Mixed experiences with commercial calibrators and controls for COVID-19 drugs

Running title: Calibrators and controls for COVID-19 drugs

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To the Editor:

In the midst of the pandemic caused by the new coronavirus SARS-CoV-2, medical scientists and practitioners are scrambling to develop and test effective therapies against the corresponding disease (COVID-19). Two of the experimental therapies include the administration of the repurposed drugs hydroxychloroquine (Plaquenil®, Sanofi-Aventis, France) (*1*) and remdesivir (Veklury®, Gilead Sciences Inc., USA) (*2*). As severe cases of COVID-19 in intensive care units often develop complications like renal insufficiency (*3*), individually adjusted drug dosing may be difficult. To avoid under- or overdosing, therapeutical drug monitoring (TDM) of these agents is very desirable. With this in mind, in our laboratory we established validated methods for the quantification of hydroxychloroqine (HPCL-UV) as well as for remdesivir and its active metabolite GS-441524 (LC-MS/MS) in human plasma by applying previously published procedures (*4, 5*). In short, samples for hydroxychloroquine analysis were prepared by addition of 0.1% trifluoroacetic acid (TFA) and 2.5 M perchloric acid as protein precipitation reagents. No internal standard was used, as no extraction losses were observed. Separation took place on a Zorbax SB-C18 150×2.1 mm column (Agilent Technologies, Germany) by gradient elution (starting at 95% 0.1% TFA and 5% methanol and evolving in 9 min to 50% 0.1% TFA and 50% methanol, flow rate 0.4 mL/min) and detection at λ = 343 nm. The retention time for hydroxychloroquine was 9.2 min. No interferences from endogenous compounds or other COVID-19 drugs in the commercial samples (lopinavir, ritonavir, favipiravir, chloroquine, nafamostat, azithromycin) were observed. Sample preparation for remdesivir and GC-441524 was carried out by protein precipitation with methanol and addition of the internal standard maraviroc (as recommended by the manufacturer of the COVID-19 test kits). Analytes were separated by gradient elution (100% ammonium formate buffer pH 3.5, evolving in 12 min to 30% buffer and 70% acetonitrile, flow rate 0.5 mL/min) on a ReproSil Pur C18-AQ 150×3 mm column (Dr. Maisch GmbH, Germany) and positive ESI detection was used. The fragment ions m/z 603→200 for remdesivir (qualifier ion: m/z 603→402), m/z 292→147 for GS-441524 (qualifier ion: m/z 292→265) and m/z 520→117 for the internal standard were observed. The corresponding retention times were 14.0, 8.6 and 11.5

min. Extraction yields were 89.4%, 86.1% and 88.2% for remdesivir, GS-441524 and the internal standard, and matrix effects were 11.3%, -1,7% and -3.0%, respectively. Calibration functions in these methods were linear in ranges of 50 – 2000 ng/mL for hydroxychloroquine and 25 – 2500 ng/mL for remdesivir and GS-441524, respectively. We achieved precision and accuracy levels at the lower limit of quantification of 1.25% relative standard deviation and 5.39% deviation from the expected concentration for hydroxychloroquine. The corresponding values for remdesivir were 2.90% and -10.87% and for GS-441524 3.71% and 8.92%.

External quality control is an important tool to check the precision and accuracy of methods in TDM. Thus, we applied a recently marketed kit set for quality control and calibration of COVID-19 drugs, including hydroxychloroquine as well as remdesivir and GS-441524 (Chromsystems Instruments & Chemicals GmbH, Germany; article numbers #0268 (plasma control level I), #0269 (plasma control level II) and #92055 (multilevel plasma calibrator set)). These kits come with an application note recommending a ready-for-use LC-MS/MS procedure developed and marketed by Chromsystems, which applies internal standards used from their anti-HIV-drug kit (article number #92844). The commercial calibration and control samples were reconstituted and stored according to the manufacturer's instructions. Applied to our quantification methods, the test kits produced the concentration values summarized in Table 1.

As can be seen, the measured values for hydroxychloroquine were near the expected values within less than 8% difference. Furthermore, all differences pointed in the same direction, implying a minor calibration offset between our in-house produced calibration samples (spiked drug-free human plasma based on hydroxychloroquine-sulfate, purity > 98%, Sigma-Aldrich, Germany) and the commercial ones. On the other hand, for remdesivir and its active metabolite GS-441524 the differences were inhomogeneous, much larger, and clearly out of an acceptable range. These deviations from our in-house calibration (spiked drug-free human plasma based on remdesivir, purity > 99%, AdipoGen life sciences, USA and GS-441524, purity > 98%, Cayman Chemicals, USA) cannot be attributed to calibration offsets because of their inhomogeneity. In turn, it was not possible to construct linear calibration functions from the Chromsystems multilevel calibrator kit for remdisivir or GS-441524 or to reproduce the expected concentration

values of the Chromsystems control kit samples. This finding, together with the fact that our in-house calibration functions for these substances were linear, contributed to severe doubts about the quality and reproducibility of these kits. A stability problem in the commercial calibration and control samples can be ruled out as explanation for the obtained results. The manufacturer warrants a shelf life for nearly two years at 2-8 °C and stability for 1 month at -18 °C for the reconstituted samples. These conditions were met in our laboratory during this investigation. Furthermore, interferences from other substances in the commercial samples were not observed as they were chromatographically separated from the here investigated analytes.

The here evaluated calibrator- and control kits are useable for the external quality control of hydroxychloroquine but not for remdesivir and its active metabolite GS-441524. Generally, the kits are not applicable for use in TDM as they are described in the manufacturer's leaflet as "for performance evaluation only" and do not bear a CE-marking which in the European Union demonstrate conformity with the requirements regarding medical products. A limitation of this study is that there are no independent third party control samples for remdesivir or GS-441524 available. Thus, we are unable to prove that our analytical method reproduces the true concentrations of the here investigated control samples.

Note: *The Journal of Applied Laboratory Medicine* editorial office invited Chromsystems to submit a Reply to this letter but received no response.

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Table 1: Expected and measured values for COVID-19 drugs from the commercial control- and calibrator kits.

Sample	Hydroxychloroquine			Remdesivir			GS-441524		
	Expected value *** (ng/mL)	Measured value (ng/mL)	Difference (%)	Expected value *** (ng/mL)	Measured value (ng/mL)	Difference (%)	Expected value *** (ng/mL)	Measured value (ng/mL)	Difference (%)
Control Level I	463	500	8.0	727	916	26.0	35.3	74.1	109.8
Control Level II	943	976	3.5	2131	1463	-31.3	67.4	99.1	47.1
Calibrator 0	0	n.d.*	n.a.**	0	n.d.	n.a.	0	n.d.	n.a.
Calibrator 1	470	506	7.7	85.2	102	19.6	22.2	48.9	120.2
Calibrator 2	689	734	6.5	1282	2331	81.8	44.3	186.7	321.5
Calibrator 3	1154	1202	4.1	2729	2984	9.3	93.4	265.8	184.6

* n.d. = not detected

** n.a. = not applicable

*** Expected values taken from manufacturer's data sheet