

# Validated Microwell-Based Spectrofluorimetric Method for Quantification of Ravidasvir (New Anti-Chronic Hepatitis C Virus-GT4) in Rat Plasma and Its Application to Pharmacokinetic Study

This article was published in the following Dove Press journal:  
*Drug Design, Development and Therapy*

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**Background:** Ravidasvir (RAV) has been regarded as a potent new NS5A inhibitor with a magnificent safety and tolerability in the management of genotype 4 hepatitis C virus (HCV) patients. Suitable analytical techniques are needed for the measurement of RAV in different biological matrices.

**Methods:** We have developed a fast, sensitive and economical 96-microwell-based spectrofluorimetric technique combined with one-step protein precipitation extraction strategy for the measurement of RAV in rat plasma.

**Results:** Under the optimum conditions, the direct relationship in rat plasma was accomplished between the RAV concentrations and the fluorescence (FL) intensity in a scope of 2.5–200 ng/mL with 0.9998 and 0.9999 for the quantification and correlation coefficients, respectively. The lower limit of detection (LLOD) was 0.840 ng/mL and this demonstrates the high sensitivity of the proposed assay. The accuracy (RE%) ranged from 95.34% to 102.29%, and the precision (RSD%) was less than 3.59%. The recovery was ranged from 93.12% to 96.26%. The stability of RAV in rat plasma was carried out and established its good stability in the range of room conventional temperature and at long-term stability (–80°C, 30 days). The developed technique was validated as stated by the United States Food and Drug Administration (US-FDA) guidelines for bioanalytical technique verification.

**Conclusion:** The approved technique was effectively applied for a pharmacokinetic (PK) study after single oral gavage administration of RAV at a dose of 35 mg/kg and it could be presumed that the proposed assay can be applied to clinical trials.

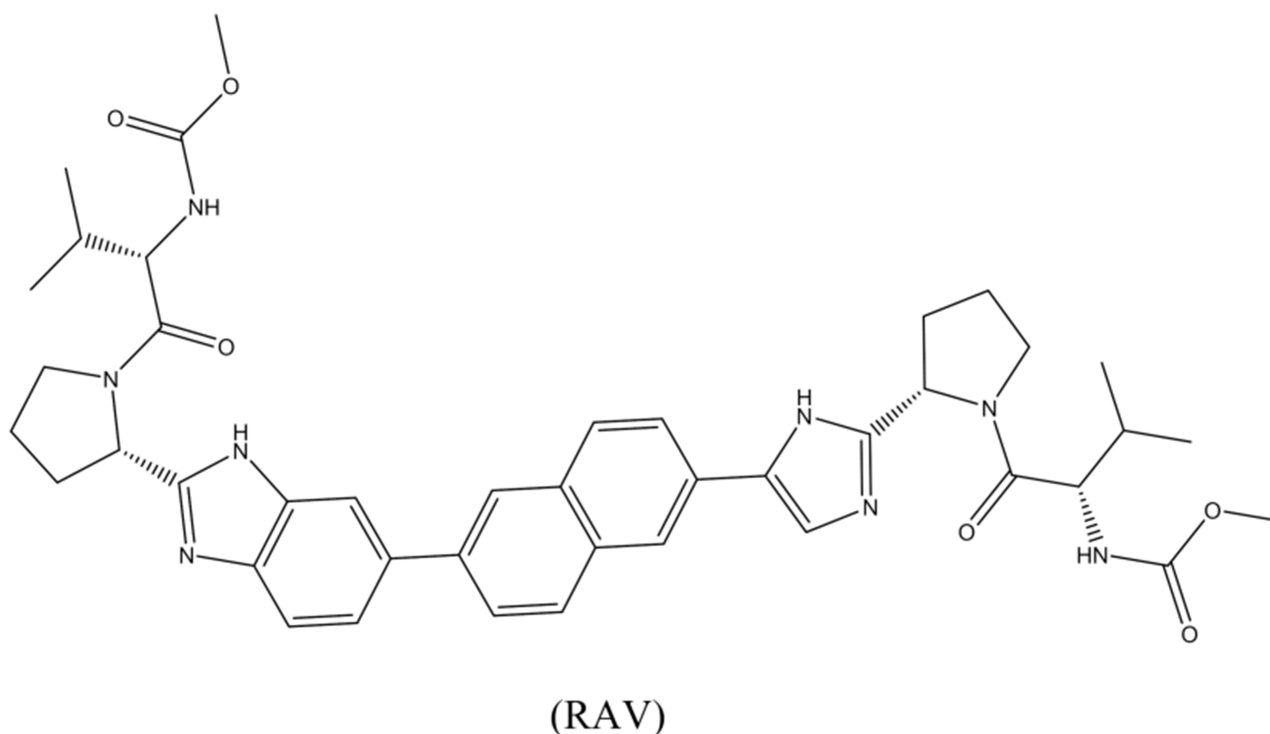
**Keywords:** spectrofluorimetry, ravidasvir, rat plasma, pharmacokinetic study

## Introduction

Hepatitis C virus (HCV) is a significant international health issue influencing 70 to 100 million individuals around the world.<sup>1</sup> HCV-genotype-4 (GT4) has been regarded as a widely common variation of HCV in the Middle East and Africa and is responsible for more than 80% of these viral cases. At a recent time, HCV has extended to various European nations.<sup>2</sup> HCV-4 has been regarded as a major cause of liver cirrhosis, chronic hepatitis, hepatocellular carcinoma, and liver transplantation.<sup>3,4</sup>

Ravidasvir (Figure 1) is chemically described as methyl N-[1-[2-[5-[6-[2-[1-[2-(methoxycarbonylamino)-3-methylbutanoyl]pyrrolidin-2-yl]-3H-benzimidazol-5-yl]naphthalen-2-yl]-1H-imidazol-2-yl]pyrrolidin-1-yl]-

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**Figure 1** Chemical structure of ravidasvir (RAV).

3-methyl-1-oxobutan-2-yl] carbamate dihydrochloride and is an investigation NS5A inhibitor that are presently experiencing clinical preliminaries for chronic HCV-4.<sup>5</sup>

NS5A inhibition has been associated with a significant reduction in HCV RNA levels in cell culture-based models, thereby placing this agent among the most potent antiviral molecule developed to date.<sup>5</sup> As of late, the World Health Organization (WHO) has included ravidasvir as a future pan-genotypic direct-acting antiviral to the list of suggested treatments in their rules for the consideration and treatment of individual detected to have HCV.<sup>6</sup> Furthermore, the Chinese Food and Drug Administration included hepatitis C NS5A inhibitor ravidasvir together with the list that was proposed as preference report to quicken its new medication application procedure.<sup>7</sup> Ravidasvir plus sofosbuvir has shown excellent efficacy and safety in genotype 4 HCV-infected adults in Egypt.<sup>8</sup>

Pharmacokinetic (PK) information shows the time course for drug concentrations to go round and are viewed as significant in the assessment of new biotherapeutics in drug improvement. In the beginning phases of analyte uncovering, preclinical PK research was usually done in animal models. The various sorts of sampling are accessible, for example, conventional composite examining, which is regularly utilized where time focuses over a period course

is checked in various animals, to get an adequate volume of information for the bioanalytical survey.<sup>9</sup> Sequential sampling for PK is considered as an advantageous methodology utilized in bioanalytical research centers.<sup>9</sup> The ethical approval of this study was approved by the Ethics Committee of the Experimental Animal Care Society, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Because of the unpredictable idea of the matrix (regularly plasma or urine) and the requirement for high affectability to take note of concentrations after low dose administration over a significant time, PK is frequently examined utilizing the fluorescence spectrometry procedure.<sup>10–12</sup> Presently, there is an interest for the utilization of high sensitivity fluorescence spectrometry for microdosing analysis, which is viewed as a promising option in substitute to animal investigation.<sup>10,11</sup>

Pilot research in our lab indicated the native fluorescence property of RAV. The additional evaluation of this occurrence prompted the recommendation that the utilization of fluorescence spectrometry could be one of the suitable choices for the quantification of RAV in rat plasma. Fluorescence spectrometry is an exceptionally quick, efficient, selective, and profoundly responsive procedure for evaluation of drug in plasma.<sup>13–16</sup> Extensive literature review revealed that report describing an analytical

technique for the measurement of RAV in biological matrix does not exist. Thus, the aim of the present investigation is to create and validate an eco-friendly, selective, high-throughput, and extremely precise method for the measurement of the RAV in plasma depends on the estimation of its native fluorescence in ethanol. The developed technique included non-extractive protein precipitation methodology combined with an ultrasound, which described by high recovery with no requirement for sample preconcentration step. The developed technique was effectively applied to the investigation of RAV in PK research in the rat. The developed procedure was validated as per US-FDA rules.<sup>17</sup>

## Materials and Methods

### Materials and Reagents

Reference standard ravidasvir (RAV) (purity > 99%) was obtained from Pharco Pharmaceutical Company (Alexandria-Cairo Desert Rd. Km 31, Amriya, Alexandria, Egypt). HPLC grade solvents, acetonitrile, methanol, ethanol, isopropanol, were supplied from the Merck Company (Darmstadt, Germany). Dimethyl sulfoxide (DMSO), NaOH and HCL were bought from Panreac Química SLU (Barcelona, Spain), and the other reagents were all analytical grade. Ultrapure water was gotten from the Ultrapure water Milli-Q Advantage water purification system, 0.22µm filter (Millipore, Molsheim, France). Healthy male Wistar rats weighing  $250 \pm 30$  g from the experimental animal care center in the College of Pharmacy at King Saud University, Saudi Arabia.

### Analytical System

Fluorimetric measurements were performed using a Microplate/cuvette reader (Spectramax M5, Devices, California, USA). Both the excitation and emission monochromator slit width were set at 1.5 nm. Wavelength calibration was carried out by estimating the emission at 380 nm after excitation at 270 nm. Molecular Probes<sup>®</sup> black-walled, clear-bottom 96-well microplates for fluorescence-based assays were gotten from Thermo Fisher Scientific UK Limited (United Kingdom). Finn pipette adjustable 8 channel-pipettes were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Milli-Q water purification system (Labo, Millipore Ltd., Bedford, USA).<sup>18</sup>

### Preparation of Standard Solution

The stock solution (100 µg/mL) of RAV was set up by dissolving 5 mg of the standard powder in 50.0 mL ethanol.

A solution of light-protected for at least 4 weeks was made stable at a temperature of 4°C. The working solution (2 µg/mL) of RAV was carried out by diluting stock solution (1 mL) in 5.0 ethanol. The eight serial dilution preparation was carried out by dilution the appropriate volumes of the working solution with ethanol in other to achieve the concentration ranges of 1.5–200 ng/mL.

## General Procedure

### Construction of Calibration Curves

An accurately measured volume (20 µL) of eight serial dilutions of RAV containing 2.5–200 ng/mL was transferred into the black-walled, clear-bottom 96-well microplates (polystyrene). The solutions were completed to 200 µL with ethanol. The resulting intensities solutions of FL were estimated at  $\lambda_{ex}/\lambda_{em}$  equal to 270/380 nm by the microplate/cuvette reader. Similarly, blank wells were processed by the utilization of 20 µL of ethanol rather than RAV. The fluorescence intensities of blank wells were subtracted from those of standard wells. Consequently, the corresponding regression equation was derived.

### Plasma Sample Preparation

Aliquots of 50 µL drug-free rat plasma samples were dispensed into Eppendorf tubes spiked with 20 µL standard solutions of RAV (2.5–200 ng/mL) and vortexed for one min. To each solution, acetonitrile of about 930 µL was included and mixed using a vortex mixer for 3 min, and this was centrifuged for 6000 rpm for 30 min.<sup>19–21</sup> Aliquots of 20 µL of the clear supernatant was conveyed to a 96-microwell plate and completed to 200 µL with ethanol to obtain RAV concentration within the calibration curve of 2.5–200 ng/mL. A blank test was completed at the same time excluding the drug.

### Method Validation

Completely procedure approval strategy for this developed FL method, including precision, accuracy, linearity, selectivity, percentage recovery, dilution integrity and stability, were compiled according to the US-FDA rules for bioanalytical procedure.<sup>17</sup>

The selectivity of the technique was ascertained by examining six different batches of rat plasma from six distinct animals, including one without analyte (blank samples), and LLOQ samples show the absence of any interfering peak from endogenous plasma at the emission wavelength of the RAV. The LLOQ (the lowest concentration of calibration curve) was set as the concentration with

accuracy between 80% and 120% and precision <20% of the RSD of the hypothetical value.<sup>17</sup>

The linearity of the developed assay was assessed from the calibration curve of RAV prepared in duplicates. At the beginning and the end of analytical batch, the calibration standards were analyzed independently. The calibration curves were developed in rat plasma by plotting the FL intensity as a function of the relating concentrations. The concentrations used 2.5, 5, 10, 50, 100, 150, 175 and 200 ng/mL for RAV. The LLOD was ascertained by establishing the minimum concentration at which the analyte can dependably be detected, utilizing the relation  $3.3(S_a)/b$ . The LLOQ was defined as the minimum level at which the analyte can be quantized, using the relation  $10(S_a)/b$ , at which the precision was not more than 20% of the RSD, and the accuracy (RE %) was within  $\pm 20\%$ . Linearity is shown by the squared correlation coefficient ( $r$ ), was assessed by computation of a least-squares regression line.

Concerning accuracy and precision assessment, the five different QC levels, namely, the lower LOQ (LLOQ) and LQC, LMQC, MQC, and HQC, respectively, at 2.5, 7.5, 50, 100, and 180 ng/mL for RAV, were utilized by investigating six replicates of each QC level on three separate days to assess the precision and accuracy of the assay. RSDs were calculated for both intra- and inter-day precisions.

Extraction recovery of RAV from present protein precipitation method was assessed by contrasting prepared samples (HQC, MQC, and LQC,  $n = 6$ ) to the reference RAV solutions in blank rat plasma extracted at identical concentrations. The extraction recovery results were expressed as a percentage (%).<sup>17</sup>

Dilution integrity was assessed by dilution of highly concentrated plasma samples, with concentrations above the linear range of the developed assay, for its impact on drug recoveries. Plasma samples spiked with high concentrations of 300 ng/mL for RAV were utilized after dilution with blank plasma samples, dilution folds (1:2 and 1:4). Diluted samples were then proceed as mentioned above.

The RAV stability was assessed at the LQC and HQC levels in six replicates utilizing the newly calibration curve. The stability of the stock solutions was tested by measuring the concentrations of the analyte after storage at  $-80^\circ\text{C}$  for 30 days. The examination of RAV stability in a plasma sample was done under the same condition as the study sample. Freeze-thaw stability was investigated by exposing the QC samples to three freezes ( $-80^\circ\text{C}$ ) and thaw (room temperature) cycles. The QCs were exposed to room temperature for 6 h and stored at  $-80^\circ\text{C}$  for

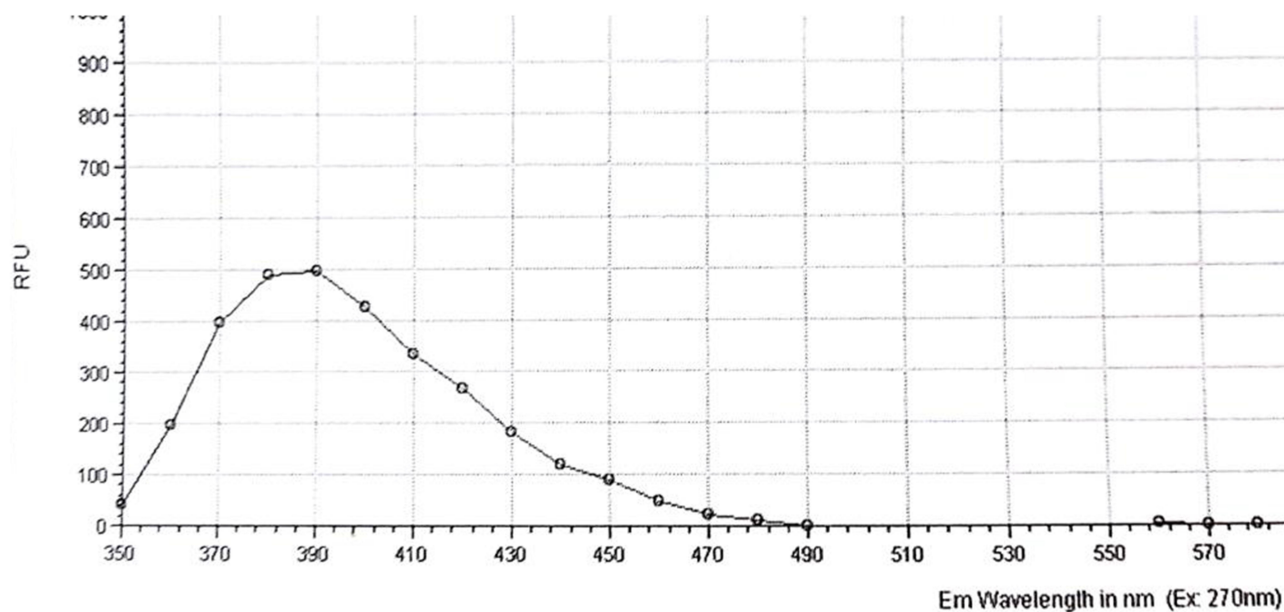
30 days for the short- and long-term stability tests, respectively. The stability of a sample is considered to be steady if the assay values were within the adequate limits of precision ( $\leq 15\%$  RSD) and accuracy ( $\pm 15\%$  SD).<sup>17</sup>

## Pharmacokinetic Study in the Rats

Four Wistar healthy male rats, weighing  $250 \pm 30$  g, were orally administered RAV (35 mg/kg) by gavage. In the current study, RAV was dissolved in 1% dimethyl sulfoxide/saline. Blood tests (approx. 300  $\mu\text{L}$ ) were gathered before drug administration, and at 30 min, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h in the wake of dosing in lithium heparinized tubes, which were put in an ice-water bath.<sup>22</sup> Plasma was isolated by centrifugation for 10 min at 3500 rpm at  $4^\circ\text{C}$  and stored at  $-80^\circ\text{C}$  till further examination. All animal experiments were with the rules of the National Institutes of Health (NIH, Bethesda, MD, USA) and the legitimate necessities in Saudi Arabia for experiments with animals and humans. The study design was approved by the Animal Ethics Committee of the Pharmacology Department, College of Pharmacy, King Saud University, Kingdom of Saudi Arabia (No. KSU-SE-18-18). Animals were housed in cages in a well-ventilated room and subjected to a regular 12 h day-night cycle at a relative humidity of 40–60% and an average temperature of  $24\text{--}27^\circ\text{C}$ . Before drug administration, rats had free access to water while the diet was forbidden for 12 h. Also, before the experiment was directed all rats were adapted for 7 days to laboratory conditions. The non-compartmental model has been utilized to evaluate the PK parameters using WinNonlin software (Pharsight Co., Mountain View, CA, USA). Area under the curve, zero to last and infinity ( $\text{AUC}_{0-24}$ ;  $\text{AUC}_{0-\infty}$ ), the maximum plasma concentration ( $C_{\text{max}}$ ), time to achieve this ( $T_{\text{max}}$ ), elimination rate constant ( $K_{\text{el}}$ ), half-life ( $T_{1/2}$ ) and mean residence time parameters were calculated. Mean, standard deviation and % RSD have been evaluated by utilizing MS-Excel 2013 (Microsoft).

## Results and Discussion

RAV has strong native FL in ethanolic solution at an emission wavelength of 380 nm after excitation at 270 nm (Figure 2). The chemical structure of RAV has various fluorophores, for example, naphthalene, imidazole and/or benzimidazole and a high degree of expanded conjugation, this was ascribed presumably to its native FL (Figure 1).<sup>22</sup> Therefore, the development of a spectrofluorimetric assay for quantification of RAV in biological fluids based on its native FL was practically possible. The proposed method was created to



**Figure 2** Fluorescence spectra of RAV (50 ng/mL) in ethanol at (25 ± 2°C).

utilize 96-microwell plate as the fluorescence response of the assay plate (200  $\mu$ L total volume). The solutions were dispensed by 8-channel pipette, and the FL signal was estimated by 96-microwell-plate fluorescence reader.

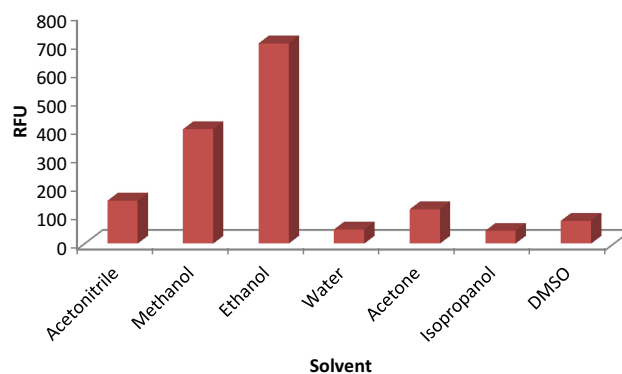
## Optimization of the Assay Conditions

### Effect of pH

The effect of pH on the FL was estimated using 0.1 M HCl, 0.1 M NaOH in addition to 0.1 M Britton–Robinson buffer solution covering the pH range 2–12. The utilization of neither 0.1 M HCl nor 0.1 M NaOH and buffer improve the FL of RAV. This survey showed that the ideal RFU of RAV was accomplished without a need to pH modification.

### Effect of Diluting Solvent

To choose the most proper diluting solvent for the proposed technique, the FL response was done in various solvents. The examined solvents were; acetone, acetonitrile, distilled water, methanol, ethanol, isopropanol, and DMSO. The maximum FL intensity performed with ethanol, which is considered as the green solvent (Figure 3). Along these conditions, the proposed technique is viewed as environmentally safe. The impact of various organic solvents on the fluorophore relying upon the relative strengths of the solute–solvent and solvent–solvent interactions. Additionally, the dissolvable molecules are typically non-symmetric dipoles and display diverse relaxation rates along their various axes. Above all, intermolecular



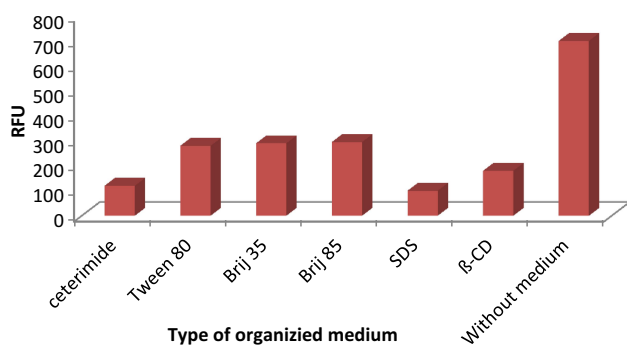
**Figure 3** Effect of diluting solvent on RFU of RAV (80 ng/mL).

adjustments of the fluorophore, the arrangement of specific complexes in the excited state and reorganization of fluorophore dissolvable hydrogen bonds may bring about temporal shifts of fluorescence spectra.<sup>23</sup>

### Effect of the Organized Media

In a preliminary to increase the FL intensity of RAV in an ethanol solution, different organized media were considered utilizing nonionic (Tween-80, Brij 35, Brij 58), cationic (cetrimide), anionic (SDS), surfactants and beta-cyclodextrin. Two mL of each composed medium (1% w/v or v/v) were added to RAV solution. It is evident from the outcomes that the presence of surfactants brought about diminished FL of RAV (Figure 4). In this manner, no surfactant was utilized in this investigation.





**Figure 4** Effect of different organized media on RFU of RAV (80 ng/mL).

### Effect of Time

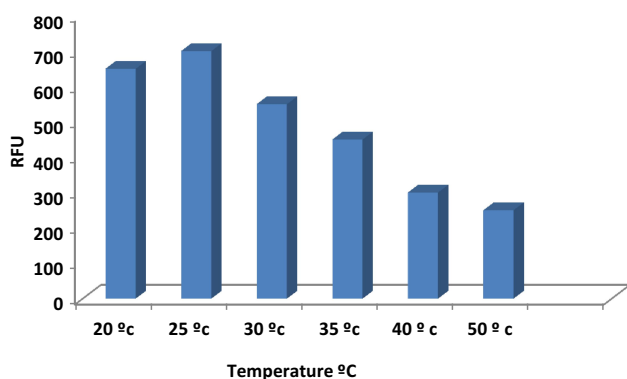
The impact of time on the native FL intensity of RAV in the ethanolic solution was reviewed from 5 min to 3 h. It was seen that the FL strength stayed stable for more than 2 h.

### Effect of Temperature

The impact of temperature on the FL strength of RAV in the chosen media was tested at various temperatures (20, 25, 30, 35, 40, and 50°C) in a thermostatically controlled water bath for 5 min. The outcomes demonstrated that room temperature (25 ± 2°C) was a perfect choice and higher temperatures were found to diminish the FL intensity (Figure 5). These impacts might be credited to collisions between the energized singlet state and the dissolvable molecules as the temperature increases.<sup>23</sup>

### Method Validation

The developed technique was approved by US-FDA rules for the bioanalytical validation approval.<sup>17</sup>



**Figure 5** Effect of temperature on on RFU of RAV (80 ng/mL).

### Linearity, and Lower Limits of Detection (LLOD) and Quantification (LLOQ)

Under the ideal conditions, the calibration graph for the quantification of RAV in pure form was created by plotting the FL intensity as a function of the corresponding concentrations. The regression equation for the results was  $y = 8.292x + 20.856$ , where  $y$  is the FL intensity at 380 nm,  $x$  is the concentration of RAV in ng/mL in the range of 1.5–200 ng/mL. The determination coefficients ( $r^2$ ) and correlation coefficients ( $r$ ) were 0.9998 and 0.9999, respectively. A summary of the statistical parameters is shown in Table 1. In case of biological samples, the calibration curve in rat plasma was built utilizing eight diverse concentration levels extended from 2.5 to 200 ng/mL, and the acquired regression equation was  $y = 7.264x + 24.981$  with the 0.9997 and 0.9994 for  $r$  and  $r^2$  respectively. An outline of the statistical parameters is introduced in Table 2.

LLOD and LLOQ of the developed FL method were calculated according to the US-FDA rules for the bioanalytical method validation.<sup>17</sup> The LLODs of the RAV were 0.47 and 0.84 ng/mL for the standard drug solutions and rat plasma, respectively. LLOD was calculated by setting up the minimum concentration at which the drug can dependably be detected, utilizing the relation  $3.3(S_a)/b$ . The LLOQ was determined as the lowest amount of drug in a sample that can be determined with satisfactory accuracy and precision under the stated experimental conditions and was seen as 1.42 and 2.54 ng/mL utilizing the relation  $10(S_a)/b$  (Tables 1 and 2).

**Table 1** Validation Data for Analysis of Ravidasvir in Pure Form Using Spectrofluorimetry

Parameters	Results
Linear range (ng/mL) <sup>a</sup>	1.5–200
$\lambda_{ex}/\lambda_{em}$ (nm)	270/380
Number of determinations	8
Intercept (a)	20.856
Slope (b)	8.292
Determination coefficient ( $r^2$ )	0.9998
Correlation coefficient ( $r$ )	0.9999
$S_a$	1.181
$S_b$	0.046
$S_{y/x}$	2.338
LLOQ (ng/mL)	1.424
LLOD (ng/mL)	0.470

**Note:** <sup>a</sup>Mean of six determinations.

**Table 2** Validation Data for Analysis of Ravidasvir in Rat Plasma Using Spectrofluorimetry

Parameters	Results
Linear range (ng/mL) <sup>a</sup>	2.5–200
$\lambda_{ex}/\lambda_{em}$ (nm)	270/380
Number of determinations	8
Intercept (a)	24.981
Slope (b)	7.264
Determination coefficient ( $r^2$ )	0.9994
Correlation coefficient (r)	0.9997
$S_a$	1.850
$S_b$	0.051
$S_{y/x}$	2.918
LLOQ (ng/mL)	2.546
LLOD (ng/mL)	0.840

Note: <sup>a</sup>Mean of six determinations.

## Precision and Accuracy

The accuracy of the proposed method was tested for intra-day (n = 6) and inter-day (n = 18) by replicating investigation of the examined drug in rat plasma at three distinct concentrations along with their direct range. Data of this are shown in Table 3. The RSD (%) for intra-day accuracy was seen as  $\leq 2.75$ , while the RSD (%) for between-day accuracy was  $\leq 3.59$ . The intra-day accuracy (%) ranged from 95.34% to 101.75%, whereas the between-day precision extended from 95.06% to 102.29%. The outcomes for exactness and precision fall into the range acknowledged by US-FDA rules for the bioanalytical method validation.<sup>17</sup>

## Extraction Recovery

The average extraction recoveries were ascertained utilizing six replicates of QC tests at three concentration levels (LQC, MQC and HQC, n = 6) in rat plasma are presented in Table 4. Recovery values of at least 93.12 ( $\pm 2.41$ ) and the RSD (%) were seen as  $\leq 1.15$ . These results demonstrated the suitability of the developed technique for the determination of rat plasma containing RAV with no matrix interference.

## Specificity/Interferences

The specificity of the technique was surveyed by analyzing ethanol solvent alone and drug-free plasma rat. The data confirmed no interference from ethanol or plasma constituents in the emission wavelength of the RAV. Furthermore, the impact of sofosbuvir, which has a combined form with RAV,<sup>8</sup> on the fluorescence

**Table 3** Intra-Day and Inter-Day Precision and Accuracy for Determination of RAV in Rat Plasma by the Developed FL Method

Analyte	Conc. Added (ng/mL)	Conc. Found (ng/mL)	Precision (RSD %) <sup>a</sup>	Accuracy (RE %)
Intra-day assay (n = 6)	2.5	2.44	2.75	97.60
	7.5	7.24	1.92	96.53
	50	47.67	0.75	95.34
	100	101.75	1.12	101.75
Inter-day assay (n = 18)	180	176.82	0.86	98.23
	2.5	2.41	3.59	96.40
	7.5	7.13	2.53	95.06
	50	47.11	0.82	94.22
	100	102.29	1.32	102.29
	180	175.72	2.47	97.62

Note: <sup>a</sup>Expressed as: RSD (%) = (SD/mean)  $\times$  100.

**Table 4** Recovery of Quality Control (QC) Samples to Determine RAV Concentrations in Plasma Matrix

Nominal Concentration (ng/mL)	RAV		
	7.5 ng/mL	75 ng/mL	150 ng/mL
Mean <sup>a</sup>	7.22	69.41	136.60
Recovery (%)	96.26	94.54	93.12
SD	2.54	3.95	0.71
Precision (RSD %) <sup>b</sup>	3.71	1.15	1.89

Notes: <sup>a</sup>Mean of six determinations. <sup>b</sup>Expressed as: RSD (%) = (SD/mean)  $\times$  100.

strength of RAV was explored. In these experiments, 1  $\mu$ g/mL of sofosbuvir in ethanol was added to the plasma and afterwards, general procedure was applied. The results demonstrated that any interfering peak of sofosbuvir was detected for quantification of RAV in plasma.

## Dilution Integrity

To exhibit the dilution integrity of RAV, plasma tests were spiked with high concentrations of RAV beyond the linear

**Table 5** Evaluation of the Dilution Integrity of RAV in Rat Plasma

Analyte	Spiked Conc. (ng/mL)	Dilution Fold	RE (%) <sup>a</sup>	RSD (%) <sup>b</sup>
RAV	300	1:2	99.10	1.26
		1:4	98.31	1.92

Notes: <sup>a</sup>Mean recovery (%) of six determinations. <sup>b</sup>Expressed as: RSD (%) = (SD/mean)  $\times$  100.

**Table 6** Stability of RAV in Rat Plasma Under Different Storage Conditions

Mean Recovery (RE %) $\pm$ RSD <sup>a</sup>					
Nominal Conc. (ng/mL)	Microwell stability (15 °C, 24 hours)	Short-term stability (25 °C, 6 hours)	Refrigeration stability overnight (4 °C)	Freeze-thaw stability (-80 °C, 3 cycles)	Long-term stability (-80 °C, 30 days)
2.5	99.82 $\pm$ 1.81	98.50 $\pm$ 1.33	98.64 $\pm$ 1.83	99.35 $\pm$ 1.36	96.73 $\pm$ 1.34
200	99.34 $\pm$ 1.63	99.25 $\pm$ 1.36	99.45 $\pm$ 1.27	99.91 $\pm$ 1.22	97.83 $\pm$ 1.28

Note: <sup>a</sup>RSD of six determinations.

range of the given strategy and diluted with rat plasma (1:2 and 1:4). The general analytical procedure for spiked rat plasma (2.6.1) was carried out. In each case, the RSD (%) and mean % recoveries were calculated as expressed in Table 5. All recoveries results were within  $\pm$  2% of the nominal values with % RSD  $\leq$  1.92 demonstrating the integrity of the RAV up to four times dilution of plasma samples.

### Stability Studies in the Biological Fluid

The stability of RAV in rat plasma was assessed at the LQC and HQC levels in triplicate all through investigation strategies by the developed FL technique. The stability tests were accompanied with for freeze-defrost cycles, bench-top stability and long-term stability. Results are outlined in Table 6. There was no noticeable decrease in the intensity of the emission wavelength of the RAV after short-term storage, three freeze-defrost cycles, long-term storage, or storage in the microwell, demonstrating that RAV displayed good stability in all conditions.

### Pharmacokinetic Study of Ravidasvir

This study was the first reported analytical method utilizing the 96-microwell-based spectrofluorimetric technique to determine RAV in rat plasma and evaluate its application to a PK

**Table 7** The Pharmacokinetic Parameters of Ravidasvir in Rat Plasma After Oral Administration 35 Mg/Kg (n = 6, Mean  $\pm$  SD)

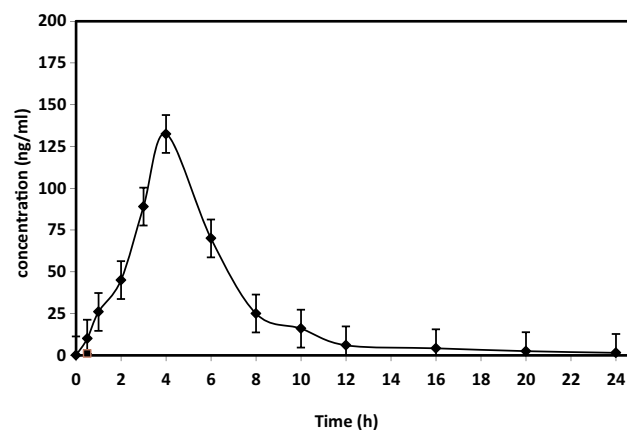
Parameters	Unit	Ravidasvir*
AUC <sub>0-24</sub>	ng/h/mL	811.25 $\pm$ 41.82
AUC <sub>0-∞</sub>	ng/h/mL	893.09 $\pm$ 44.78
C <sub>max</sub>	ng/mL	132.35 $\pm$ 14.56
T <sub>max</sub>	h	4.00 $\pm$ 0.08
Cl/F	ng/mL/h	0.0419
t <sub>1/2</sub>	h	3.24 $\pm$ 0.99
MRT <sub>0-∞</sub>	h	5.56 $\pm$ 0.76

Note: \*Data are presented as mean  $\pm$  SD.

study. Following the single oral gavage administration of RAV at a dose of 35 mg/kg to the rats, a mean peak plasma concentration (C<sub>max</sub>) of 132.35  $\pm$  12.56 ng mL<sup>-1</sup> was attained in ~4 h. The C<sub>max</sub> values lie within the working concentration range of the developed FL assay. The pharmacokinetic parameters results for RAV are summarized in Table 7. The ratios of AUC (AUC<sub>0-24</sub>/AUC<sub>0-∞</sub>) were found to be  $\geq$ 90%, demonstrating that the present FL method was sensitive sufficient to cover the elimination phase of RAV. The mean plasma concentration-time curves of RAV in rats are presented in Figure 6.

### Conclusion

This study presents a validated 96-microwell-based spectrofluorimetric method for analysis of RAV depending on the measuring of its native fluorescence in ethanol. The proposed method having many advantages, including short analysis time, simple operation procedure, high sensitivity, and selectivity. Also, it does not include any time-consuming extraction and derivatisation reactions with ease of measuring the fluorescence intensity that is established immediately. Furthermore, this study was the first analytical method utilized 96-microwell-based spectrofluorimetric technique for

**Figure 6** Concentration-time profile of ravidasvir (RAV) in rat plasma after single oral gavage administration of RAV at a dose of 35 mg/kg. Points are means  $\pm$  SD.



determination of RAV in rat plasma and its application to PK study. The developed method is characterized by high-throughput analysis, which facilitates the processing of many samples in quality control and clinical laboratories.

## Abbreviations

FL, fluorescence; LLOQ, the lower limit of quantification; LLOD, the lower limit of detection; US-FDA, United States Food and Drug Administration; PK, pharmacokinetic; QCs, quality control sample.

## Acknowledgments

The authors extend their appreciation to the Deputyship for Research & Innovation, “Ministry of Education” in Saudia Arabia for funding this research work through the project number IFKSURG-037. Also, the authors are thankful to Dr. Mohammed Abounassif (King Saud University) for his help and technical assistance.

## Disclosure

The authors report no conflicts of interest for this work.

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