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Original Article

Green Assessment of Analytical Procedure for the Determination of Anti-Retro Viral Drugs by HPLC

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ABSTRACT

Aim: The proposed study was to establish a Greener method and validation, by green chromatography technique for combined anti-viral drugs, measuring greenness using various tools, which is one of the emerging developments in the analytical field. **MATERIAL AND METHOD:** The chromatographic separation is achieved by using C_{18} (250x4.6, 5 µm) column by applying isocratic elution using the mobile phase containing Ethanol and Isopropyl acetate in the ratio of (55:45% v/v) with 1 mL/min flow rate. **RESULT:** The separation of drugs is achieved by using greener mobile phase.The Retention time of 1.854 min and 9.09 min for Ritonavir and Ombitasvir was found respectively. The regression co-efficient (R^2) is 0.987 for the both drugs. Accuracy and precision is evaluated for the method and found be within the limit and the results were reproducible. Assessment of method was carried out using the three different tools. The developed method is anticipated to be eco-friendly, alternative to developed method UPLC method in regard to safe solvent, less toxic and less run time. The Proposed method was found suitable for the simultaneous estimation in their combined dosage form.

Keywords: Green chromatography, Antiviral drugs, UFLC, Complex GAPI, AGREE, AMGS.

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INTRODUCTION

Ombitasvir, is an antiviral medication, to treat Hepatitis C virus infection and is a potent, non-structural protein 5A (NS5A) inhibitor of the hepatitis C virus that is commonly used in conjunction with other drugs to treat chronic HCV infection.[1-2]

Ritonavir has a CYP3A inhibitor of HIV type 1 and protease inhibitor for HIV virus that alters the reproductive cycle in HIV patients. It can also be used to treat COVID-19 and hepatitis in conjunction with other drugs. To improve their blood concentration, two SARS-CoV-2 3CLpro inhibitors are prepackaged with ritonavir,[3,4] both the drugs have good absorption and have (T_{max}) of approximately 4 to 5 hours. After the 12 days of dosing steady state exposures are achieved.[5] Chronic hepatitis C is an infectious liver disease caused by HCV infection that is treated with a combination of direct-acting antivirals called Ombitasvir.[6]

A new coronavirus (COVID 19) produced by the SARS-CoV-2 virus category has been identified as one of the coronaviruses belonging to the Coronaviridae family. It can cause severe fever and other respiratory disorders like pneumonia and dyspnea. On the other hand, antiretroviral medication used to treat HIV is ritonavir;lopinavir and Ombitasvir etc,is typically taken in conjunction with other antivirals that work well together as a result, it was repositioned as a medication that was given in addition to treat COVID-19.[5,6]

These days, the pharmaceutical industry's analytical division uses terms like "green chemistry,"benign chemistry," and so on. These division focus on reducing or eliminating the use of hazardous or toxic solvents, waste production, feedstock use, energy consumption, and waste generation. The main objective of these methods is to eliminate dangerous and poisonous substances and replace them with safer alternatives that are better for the environment and the analyst's health. Anastas introduced analytical chemistry to the 12 principles of green chemistry.[7]

Reducing the usage of solvent demand for sample pre-treatment, the quantity and toxicity of solvents and solvents used in the operational step are the goals of green assessment, particularly through the principle of green analytical chemistry. An easy-to-use tool that facilitates result interpretation is the Green Analytical Procedures Index (GAPI), which is based on pictograms. It is an evaluation of the analytical process that considers sampling, preparation of sample, the consumption of solvents and reagents, instrumentation, and waste generated; nonetheless, it considers a broader range of factors than other green metrics, such as NEMI. There are three grades colours in it: red, yellow, and

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green. To measure the proposed method's greenness relative to other published methods, three new techniques were adopted to measure, Green Analytical procedure index, GAPI, and AMGS Spreadsheet.[8,9]

A tool called eco-scale is semi-quantitative in nature; it measures the quantity or amount of chemicals used, the risk associated with each reagent, the power consumed by the device, and the waste generated. A perfect green analysis credits a score of 100; any deviation from this score results in a penalty point. Eco-scale results show that our method is more environmentally friendly than other published chromatography-based techniques like LC-MS and UHPLC-MS.

Moreover, a novel software called the Green Analytical Procedure Index (GAPI), Analytical Greenness (AGREE), and National Environmental Method Index tool is found to measure the overall greenness of the method in analytical procedure, from sample preparation and collection to the final estimation. The GAPI presentation is a useful tool for procedure comparison and simplifies the process of selecting the most environmentally friendly approach for a given method. The agreement between the results from the Analytical Eco-scale, GAPI tool and the AGREE evaluation method confirms the green nature of the developed analysis.[3]

MATERIALS AND METHODS

Reagents and Chemicals

Ritonavir and Ombitasvir API are procured from gift samples from Hetero Chemical, Hyderabad respectively. HPLC grade ethanol and isopropyl acetate were received from SD Fine Chem. Ltd., Mumbai-400013 and Type-I water is used in all procedure was obtained in house.

Instrumentation

A Shimadzu prominence liquid chromatograph (UFLC) model LC20AD with a UV-visible detector (model SPD20A) and an auto sampler (model SIL20AC HT) with temperature control for the column oven was used for the analysis. Software for lab solutions is used to process and interpret data.

Mobile Phase Preparation

Ethanolandisopropyl acetate are prepared by adding in the ration of 55:45 (*v/v*) and filtered using 0.45 micron membrane filter (Millipore). This mobile phase is used to make appropriate dilution from the stock solution.

Coloumselection

The selection of analytical columns in the method development is one of the most important steps, based on the nature of the sample and the type of analysis.[10] The C_{18} column is most preferred in UFLC due to its optimum resolution and good peak for the separation of drug samples. As per literature surveys, the C_{18} column is one of the most ideal and preferred for method developmentand validation.

Preparation of Standard Solution

Ritonavir and Ombitasvir[11] stock solution were prepared in a clean 100 ml volumetric flask at a concentration of 10 mg/ml and further dilution were made to individual concentrations using the mobile phase as solvent.

Ritonavir (RTV)

From the above standard solution of Ritonavir, serial dilution is performed to get the 1000ng/ml to 15000ng/ml concentration with mobile phase. A series of dilution was done to get the concentration curve which has the acceptance value.

Ombitasvir (OMB)

From the above stock solution of Ombitasvir, serial dilution is performed to produce the 1250 ng/ml to 17500 ng/ml concentration with mobile phase. A serial dilution was done to get the concentration curve which has the acceptance value.

RESULTS

RP-UPLC

In the RP-UPLC, the chromatographic condition was optimized by selecting greener and benign solvents without affecting parameters like specificity, sensitivity, or reproducibility to achieve an adequate separation of the sample mixture. Initially, by changing different mobile phase compositions and ratios, we tried to achieve the best separation. To determine the flow rate and mobile phase, Peak parameters such as theoretical plates, tailing factor, run time and resolution were used. The Standard chromatogram of Ritonavir and Ombitasvir are shown in Figure 1 and peak area and retention time is mentioned in Table 1. The optimized data is shown in Table 2.

Method Validation

Method validation as per ICH guidelines.

Specificity

For the proposed method the specificity parameter expresses the good separation of Ritonavir and Ombitasvir without any additional peaks which indicates the method is specific to drug analytes. Given chromatograms were investigated and found to be free from interference with drug substances.

Calibration Curve and Linearity of Ritonavir and Ombitasvir

To plot the calibration curve and evaluate the regression coefficient, the linearity of ritonavir and Ombitasvir was examined by taking into the concentration range of (1000 ng/

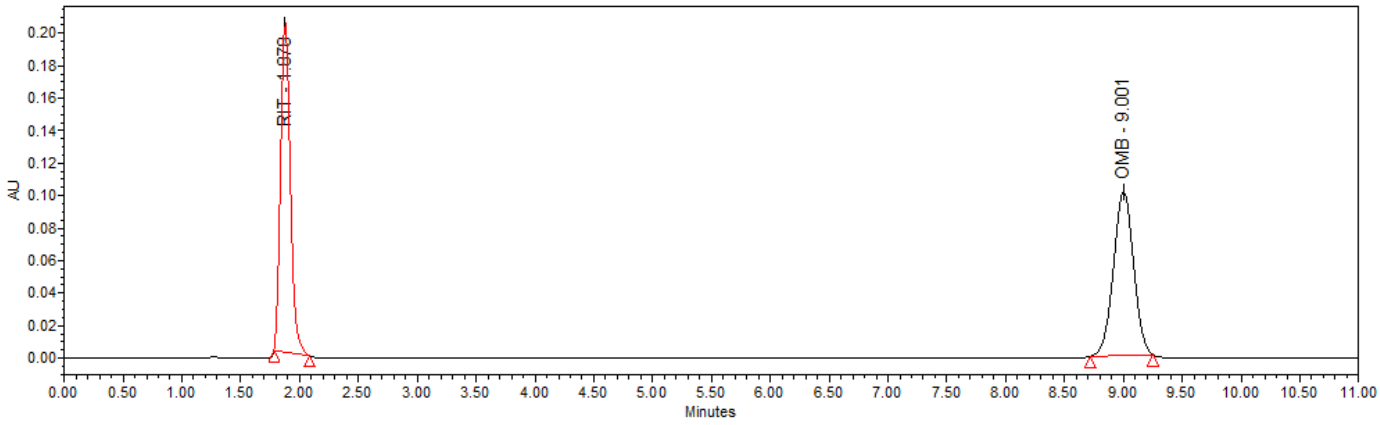

Figure 1: Standard chromatogram of Ritonavir and Ombitasvir.

Table 1: System suitability for Ritonavir and Ombitasvir.

Sl. No.	Name	Retention Time	Area	% Area	Height
1	RIT	1.870	1072448	40.47	139703
2	OMB	9.09	862455	32.54	54659

RIT: Ritonavir, OMB: Ombitasvir.

mL to 15000 ng/mL) and Ombitasvir (1250 ng/mL to 17500 ng/mL). The correlation coefficient (R^2) was consistently higher than 0.997 for all calibration curves as shown in Figures 2-3.

Observation

The linearity curve was measure for Ritonavir and Ombitasvir was generated from 1000 ng/mL to 15000 ng/mL and 12500 ng/mL to 17500 ng/mL respectively and R^2 was found to be 1.0 which is under the acceptance criteria as given in Table 3.

Accuracy

Accuracy of Ritonavir and Ombitasvir

The standard addition method was used to measure the accuracy of the developed method. The accuracy was done by taking the known amount of standard solution, three different levels of sample is spiked (50%, 100% and 150%). The study was done in triplicate and the amount of ritonavir recovered for each concentration was calculated. A direct recovery study was calculated after determining RT and peak areas. The parameter was used to assess method accuracy for developed method as shown in Tables 4-5.

Precision

Six different standard solution of RTV and OMB were analysed simultaneously at different time schedule into a UFLC. The chromatogram obtained was determine the peak area for the proposed method. The retention time and peak area of RTV and DRV were calculated and the percentage RSD was also calculated.

Table 2: Optimization of chromatographic condition.

Sl. No.	Standard concentration	Ritonavir and Ombitasvir
1.	Mobile phase	Ethanol and Isopropyl acetate
2.	Mobile phase ratio	55:45
3.	Flow rate	1 mL/min
4.	Pump	Isocratic
5.	Retention Time	1.854 and 9.09 min,
6.	Detector	UV
7.	Column Temperature	35°C
8.	Wavelength	254 nm
9.	Injection volume	20 µL

Observation

For the repeatability study, the percentage RSD for the Ritonavir Peak Area was 0.081 and the percentage RSD for Retention Time was 0.13. The method precision was found to be within 2.0% which is an acceptable limit. For the repeatability study of Ombitasvir, the percentage RSD for Peak Area was 0.106 and the percentage RSD for Retention Time was 0.80. The overall results for both drugs fall under acceptable limits which indicates that this method is precise as per the guild lines. The result table is shown in Tables 6-7.

Acceptance Requirement: The repeatability percentage RSD cannot exceed 2.0%.

Table 3: Calibration Curve and Linearity of Ritonavir and Ombitasvir.

RIT Con in ng/mL	AREA	IST AREA	RP
1000	8873	24062	0.3721
5000	45849	24063	1.9029
7500	68762	24064	2.8575
10000	90146	24065	3.7459
15000	138652	24066	5.7613
OMB Con in ng/mL	**AREA**	**IST AREA**	**RP**
1250	6985	24062	0.2903
2500	14075	24063	0.5849
5000	34985	24064	1.4538
7500	53384	24065	2.2183
17500	121789	24066	5.0606

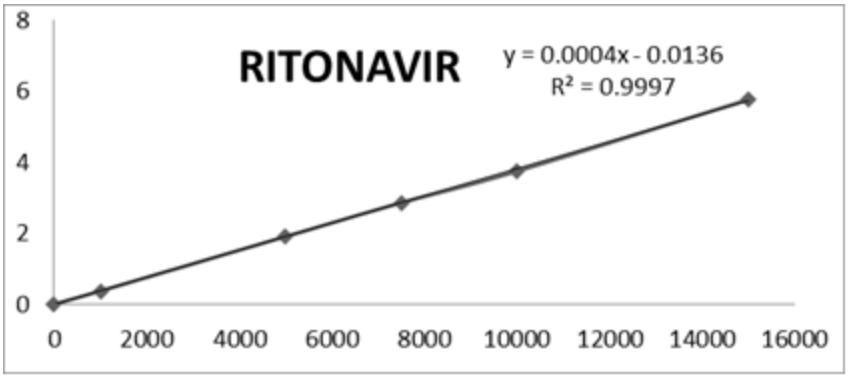

Figure 2: Standard curve data for Ritonavir.

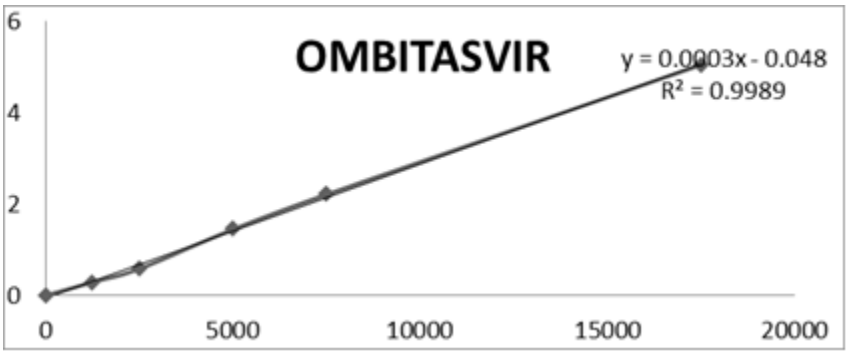

Figure 3: Standard curve data for Ombitasvir.

Limit of Detection (LOD)

The lowest concentration of analytes in a sample is detectable but not necessary to quantified in a sample for the proposed method. In chromatogram the detection limit is the injection amount that results in a peak with a height at least two or three times as high as of the baseline noise level.

Detection limit is symbolised as follow (DL).

$$DL = 3.3\ \sigma/S$$

Limit of Quantification

The Least amount of analyte in a sample indicates the quantification limit, which can be used to determine the lowest concentration of the drug with accuracy, and precision in acceptable values by the method and is especially useful for estimating the impurity profile of the drug substance (S/N ration-10).

LOD and LOQ of RTV were calculated to 300 ngm/mL and 850 ngm/mL and for OMB it was 500 ng/mL of LOD and 1000 ngm/mL of LOQ was found.

Table 4: Accuracy table of Ritonavir.

Sl. No.	Level of % recovery	Amount of drug taken (ng/mL) (STD)	Amount of drug added (ng/mL) (sample)	Peak area	Conc. found	SD	% RSD
1	50	5000	2500	53384	7500	0.62	0.20
				53384	7500		
				53384	7500		
2	100	5000	5000	67489	10190	11.5	0.11
				67469	10172		
				67478	10169		
3	150	5000	7500	82650	12540	23.4	0.18
				82580	12498		
				82586	12501		

Table 5: Accuracy table of Ombitasvir.

Sl. No.	Level of % recovery	Amount of drug taken (ng/mL) (STD)	Amount of drug added (ng/mL) (sample)	Total amount of drug (*n*=3)	Peak area	Conc. found	SD	% RSD
1	50	7500	3750	11250	91146	11250	15.2	0.13
					91240	11260		
					91258	11280		
2	100	7500	7500	15000	138752	15010	62.5	0.41
					138672	15025		
					138567	15125		
3	150	7500	11250	18750	165511	18760	10.9	0.05
					165544	18776		
					165556	18755		

Table 6: Repeatability Data Ritonavir.

Mean (Ritonavir)	1.8538	8953.5
SD	0.002	6.44
%RSD	0.13	0.081

Table 7: Repeatability Data Ombitasvir.

Mean (Ombitasvir)	8.089	6981.06
SD	0.0063	7.44
%RSD	0.080	0.106

Tablet Assay

Tablet equivalent weight of powder taken from the in-house prepared sample of Ombitasvir 12.5 mg and ritonavir 50 mg from the pooled powder of twenty tablets and transferred into a clean volumetric flask and diluted with methanol, sonicated for 10 min at ambient temperature. The sample was filtered through a 0.45-micron filter and further dilution was done for analysis the results was found to be acceptable as show in Table 8.

Green Assessment

Greening an analytical method as well as achieving the analytical parameters such as selectivity, specificity and limit of detection have a great challenging to analyst in developing the method under the green analytical chemistry.

Green analytical procedure Index: Since it assesses the greenness of every stage of the analysis process, from sample preparation to the conclusion, it is one of the most crucial tools that analysts use to evaluate the method's greenness. The evaluation criteria are

Table 8: **Assay of Tablets.**

Drugs name	Percentage Assay
Ritonavir-50 mg	97.4
Ombitasvir-12.5 mg	98.5

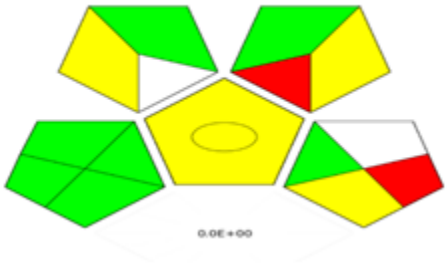

Figure 4: Complex GAPI (Green Analytical Procedure Index).

Figure 5: AGREE (Analytical GREEnness Metric Approach).

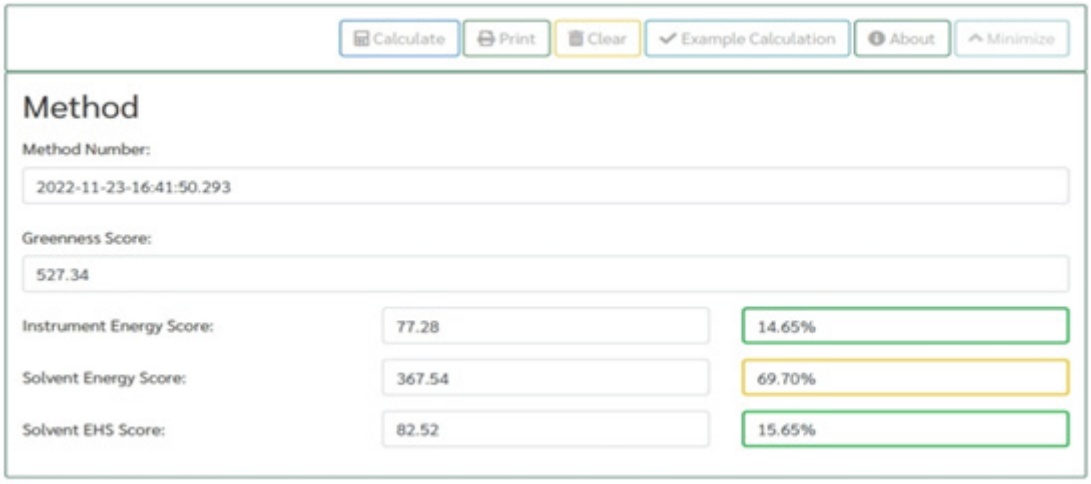

Figure 6: AMGS (Analytical Method Greenness Score) Spreadsheet calculator.

measured by taking into the things like sample size, throuSince it assesses the greenness of every stage of the analysis process, from sample preparation to the conclusion, it is one of the most crucial tools that analysts use to evaluate the method's greenness. A variety of factors are considered when measuring the assessment criteria, including sample size, throughput, waste generation, power consumption, and the choice and application of reagents, materials, and samples.[12] Another basis for assessment was the ability to distinguish between criteria based on relative relevance by awarding weights to each.[13]

The GAPI metric consists of a colour scale to a pictogram to categorise the level of "greenness" of each phase of an analytical step, with two or three levels of evaluation for each stage. Reagents, practises and equipment are assessed in GAPI. As a result, a variety of elements are taken into account, including energy needs as well as chemical health and environmental risks. In addition, GAPI provides details on the complete analytical protocol. It's crucial to note that the GAPI pictogram's small design makes it simple to compare various approaches side by side and choose the one that is most environmentally friendly for a certain study.[14-16]

The Analytical GREEness Calculator is a comprehensive, adaptable and straightforward evaluation method that produces a clear and instructive result. The evaluation parameter is based on the GAC principles and is converted into a 0-1 scale which reflect the zero as more hazardous and hight impact on environmental and one with lowest impact on system and analyst.[17]

DISCUSSION

The suggested technique has been verified as being green and environmentally friendly in accordance with ICH guidelines. It uses an eco solvent system, which can be accessed using a variety of tools and software, to verify the purity and estimation of RITONAVIR and OMBITASVIR in pharmaceutical bulk drugs.

The parameters for the RP-UFLC greener method were optimized by using different ratios of mobile phase, resulting in the best separation for eluted compounds. To separate analytes, various mobile phase compositions were initially tested by trial and error and considering the Peak parameters like theoretical plates, tailing, resolution, retention time and peak purity which judged to select the mobile phase and flow rate. The mobile phase with a ratio (55:45, v/v) of Ethanol and Isopropyl of 1.0 mL/min flow rate, indicates that the proposed method is precise and accurate.

A system suitability test was performed by taking different parameters and the test was carried out under different conditions and the results were found within acceptable limits.

The standard curve was plotted with different for concentration range for the ritonavir and Ombitasvir from 1000 ng/mL to 15000 ng/mL and 1250 ng/mL to 17500 ng/mL by linear least square analysis. The calibration curve peak area versus concentration was found to be linear and the regression coefficient (r^2) was found to be 0.9997 and 0.9989 respectively for ritonavir and Ombitasvir and percentage RSD for calibration data was found below 2, which drew the result that this proposed method was linear to the entire range which is selected for linearity study.

According to the findings, none of the interference substances/ excipients interfered with the retention time of the analytes. As a result, the proposed method is specific as per guidelines.

By taking a greater number of solutions of the same sample during the day (intraday) and the following three days (interday), the precision parameter was evaluated in terms of repeatability and repeatability was expressed in terms of %RSD. The values were on the low side when the %RSD for each case was analyzed. The percentage RSD is within the allowable range, suggesting that the approach is accurate. The limit of detection and the limit of quantification for the suggested approach were found to be sensitive when LOD and LOQ were calculated using a signal-to-noise ratio of 2:1.

Assessment of Greenness for Developed Method

Complex GAPI

The Complex GAPI is made up of five pentagrams that are used to measure and quantify the environmental impact of every step of the developed process using a different colour code: green, yellow and red which indicate a low, medium and high environmental impact. More green colour shades indicate high environmental safety and less risk to analysts.

For the developed Method, its shows eight green shaded pentagrams, four with yellow shaded and zero with Red as show in pictogram. This indicates that proposed method is very much eco-friendly and analyst safety as given in Figure 4.

AGREE TOOL

The 2nd tool is AGREE with scale range from 0-1 which indicates more greenness for 1 and 0 for the least. By keeping the above, for proposed method shows 0.83, which is also greener in the scale range of 0-1 indicating the method is eco-friendly and safe to analyst as shown in Figure 5.

AMGS TOOL

The third tool is the AMGS (Analytical Method Greenness Score), which measures the energy used by instrument score, solvent energy and solvent EHS score as 14.65%, 69.70% and 15.65%, respectively, with a total greenness score of 527.34. These colours are intended to be an indicator highlighting the method's highest contribution to the AMGS value value shown in Figure 6 indicating that the method is greener and more eco-friendly in nature, as well as safer for analysts who handled routine analysis in the quality control lab.

CONCLUSION

In this work, a novel RP-UFLC method for Ritonavir and Ombitasvir was created. It uses an eco-friendly solvent and greener chromatography. Since this approach is straightforward, exact, accurate, safer for analysts, and ecologically friendly, find to estimate ritonavir and Ombitasvir. In commercial formulations, this approach is suitable for quantifying Ombitasvir and Ritonavir. According to the AMGS spreadsheet metrics, AGREE greenness, and GAPI greenness, the suggested technique can reduce the environmental effect of other organic mobile phase solvents, such as acetonitrile, and improve safety for analysts doing regular analysis in quality control.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest.

ABBREVIATIONS

RP-UFLC: Reverse phase ultra-fast liquid chromatography; **RTV:** Ritonavir; **OMB:** Ombitasvir, **GAPI:** Green Analytical Procedure Index; **AGREE:** Analytical GREEnness Metric Approach; **AMGS:**

Analytical Method Greenness Score; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **RSD:** Relative standard deviation; **RT:** Retention time; **mg:** Milligram; **ng:** Nanogram; **mL:** Milliliter.

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