RESEARCH ARTICLE

UFLC Method Development and Validation for Anti-retroviral Drugs, Investigation of Greenness Assessment using Complex GAPI, AGREE and AMGS SPREADSHEET

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ABSTRACT

Introduction: The study's proposal was to establish a method of development and validation by green chromatography technique for antiviral drugs, as well as to assess the proposed method's greenness using various tools, which is one of the emerging developments in the analytical field. The chromatographic separation is achieved by using Eclipse plus C18 (250 x 4.6, 5 µm) column by applying isocratic elution using the mobile phase containing methanol and isopropyl acetate in the ratio of (60:40% v/v) with 1-mL/min flow rate. The separation of drugs is achieved by using a greener mobile phase.

Results: The retention time of ritonavir and ombitasvir was found to be 1.854 and 8.09 minutes, respectively. The regression coefficient (R2) is 0.997 for both drugs. Accuracy and precision is evaluated for the method and found be within the limit and the results were reproducible. Assessment of the method was carried out using the three different tools. The developed method is anticipated to be eco-friendly, an alternative to the developed method HPLC method in regard to safe solvent, less toxic and less run time. The developed method was found suitable for the simultaneous estimation in their combined dosage form.

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

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INTRODUCTION

Ombitasvir is an antiviral medication prescribed by AbbVie to cure hepatitis C virus infection. Ombitasvir is a potent, non-structural protein 5A (NS5A) that inhibits the hepatitis C virus, which is widely used in combination with other drugs for the treatment of chronic HCV infection.[1,2]

Ritonavir has a CYP3A inhibitor of HIV type 1 and protease inhibitor for the human immunodeficiency virus (HIV) that altered 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.[1,6]

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, an anti-retroviral 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 treating COVID-19.[5]

Green Chromatography

The terms "green chemistry," "clean chemistry," "benign chemistry," and so on are emerging area used in the pharmaceutical industry of analytical division emphasizing mainly to reduce or decrease the use of hazardous/toxic solvents, waste production, feedstock use, energy consumption, and waste generation. These methods aim to eliminate toxic, hazardous substances and replace them with safer alternatives that benefit for analyst health and environment. 12 The principle of green chemistry was introduced by Anastas for analytical chemistry,[7] reducing the usage of solvents demand for sample pre-treatment, the quantity and toxicity of solvents and solvents used in the operational step are the goals of

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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. It has three grade colors: red, yellow, and green. To measure the proposed method's greenness relative to other published methods, three new techniques were adopted to measure, GAPI and AMGS Spreadsheet.[8,9]

An eco-scale is semi-quantitative; 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 credit a score of 100; any deviation from this score results in penalty point. Eco-scale results show that our method is more environmentally friendly than other published chromatography-based techniques like liquid chromatography-mass spectrometry (LC-MS) and ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS).[8,10]

Moreover, a novel software called the 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.[11] 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 is procured from Yarrow Chemical, Mumbai-421201, respectively. HPLC grade methanol and isopropyl acetate has received from the SD Fine Chem Ltd, Mumbai-400013 and Type -I water is used in all procedures was obtained in-house.

Instrumentation

Analysis was conducted on a prominent liquid chromatograph UFLC of Shimadzu, model LC20AD with a UV-visible detector of model SPD20A with auto sampler model SIL20AC HT with column oven temperature control. Lab solution software is used for data processing and interpretation.

Selection of Mobile Phase

The selection of MP solvent is done by changing the mobile phase mixture in different ratios, buffer compositions and trial-and-error principles. Considering the physiochemical properties during the preparation of the mobile phase, the following criteria like, stability, solubility, pKa value and literature review.[12]

Mobile phase preparation

Methanol and isopropyl acetate are prepared by adding the ration of 60:40 (v/v) and filtered using a 0.45 micron membrane filter (Millipore). This mobile phase is used to make appropriate dilution from the stock solution

Analytical columns selection

The selection of analytical columns in the method development is one of the most important steps is based on the type of analysis and sample nature. 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 development and validation. 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. 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 development and validation.

Preparation of standard solution

Ritonavir and ombitasvir stock solution were prepared in a clean 100 mL volumetric flask at 5 mg/mL concentration. Further dilution was made to individual concentrations using the mobile phase as solvent.

Ritonavir

From the above standard ritonavir (RTV) solution, serial dilution is performed to get the 1000 to 15000 ng/mL concentration with the mobile phase. A series of dilutions was done to get the concentration curve which has the acceptance value.

Ombitasvir

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

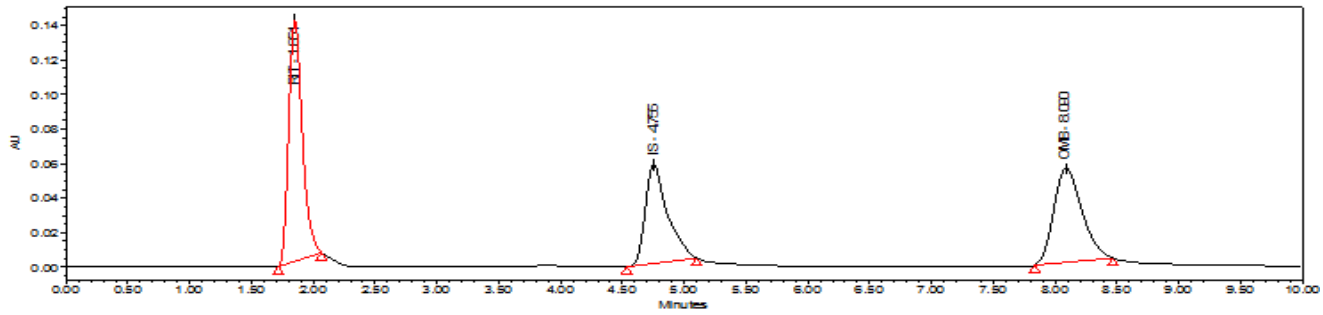
RESULTS

RP-UPLC

In the reversed-phase ultra-fast liquid chromatography (RP-UFLC), the chromatographic condition was optimized by selecting greener and benign solvents without affecting parameters like specificity, sensitivity, or reproducibility to adequately separate the sample mixture. Initially, we tried to achieve the best separation by changing different mobile phase compositions and ratios. Peak parameters such as theoretical plates, tailing factor, run time, and resolution were used to determine the flow rate and mobile phase. The standard

Table 1: System suitability for ritonavir and ombitasvir

S. No.	*Name*	*Retention time*	*Area*	*%Area*	*Height*
1	RIT	1.851	1072448	40.47	139703
2	IS	4.755	715243	26.99	57681
3	OMB	8.09	862455	32.54	54659



RIT = Ritonavir, IS = Internal standard, OMB = Ombitasvir

Figure1: Standard chromatogram of ritonavir and ombitasvir

chromatogram of ritonavir and ombitasvir accuracy are shown in Figure 1.

Method Validation

Method validation as per ICH guidelines

Specificity

For the prosed method the specificity parameter expresses the good separation of ritonavir and ombitasvir without any additional peaks which indicate method is specific to drug analytes. All the chromogram 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 (1000–15000 ng/mL) and ombitasvir (1250–17500 ng/mL). The correlation coefficient (R2) was consistently higher than 0.997 for all calibration curves.

- *Observation*

The linearity curve was measured for ritonavir and ombitasvir was generated from 1000 to 15000 ng/mL and 12500 to 17500 ng/mL, respectively and R2 was found to be 1.0, under the acceptance criteria.

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 the accuracy of the developed method.

Precision

Six standard solutions for RTV and OMB were analyzed simultaneously at different time schedules and added to a UFLC. The chromatogram obtained determines the peak area for the proposed method. The retention time and peak area of

Table 2: Optimization of chromatographic condition

S. No.	*Standard concentration*	*Ritonavir and ombitasvir*
1.	Mobile phase	Methanol and isopropyl acetate
2.	Mobile phase ratio	60:40
3.	Flow rate	1-mL/min
4.	Pump	Isocratic
5.	Retention time	1.854 and 8.09 minutes
6.	Detector	UV
7.	Column temperature	25°C
8.	Wavelength	254 nm
9.	Injection volume	20 µL

Table 3: Calibration curve and linearity of ritonavir and ombitasvir

RIT Con in ng/mL	*Area*	*Ist Area*	*RP*
1000	8953	24062	0.3721
5000	45789	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

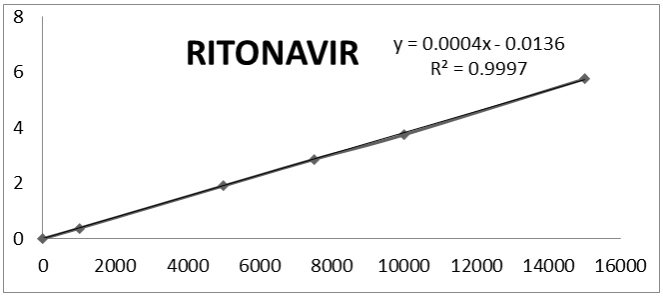


Figure 2: Standard curve data for ritonavir

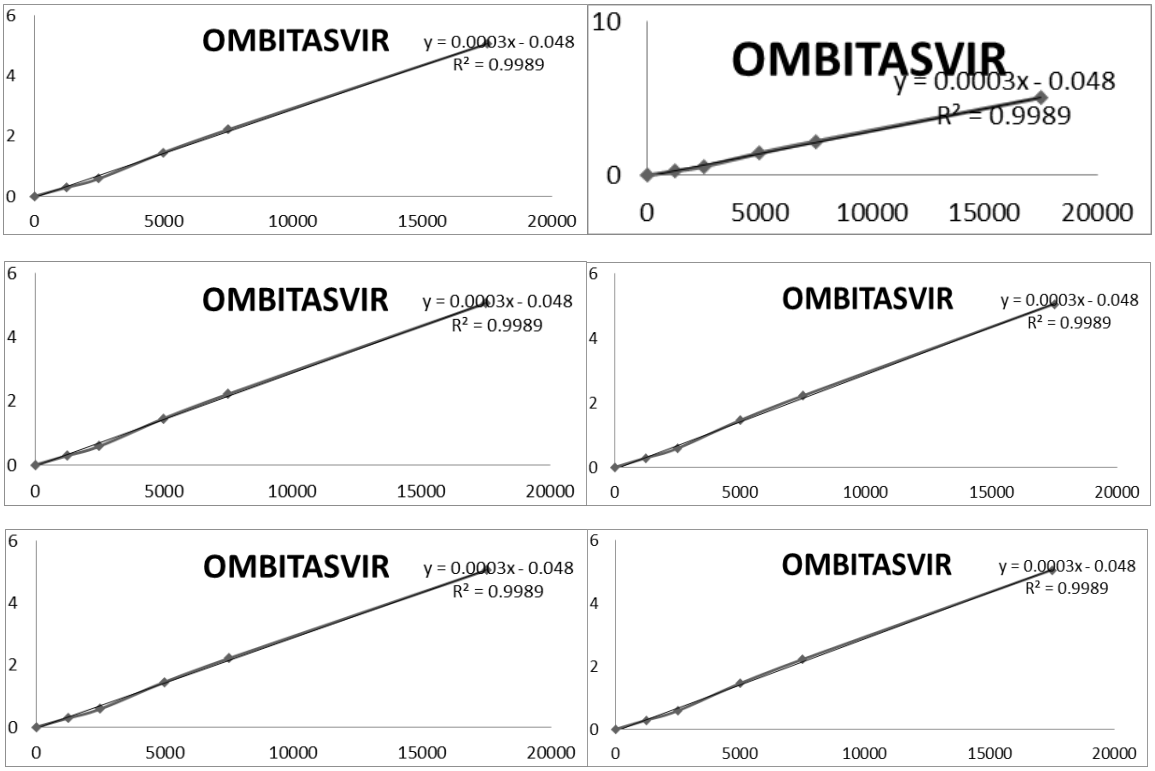


Figure 3: Standard curve data for ombitasvir

Table 4: Accuracy table of ritonavir

S. No.	*Level of % recovery*	*Amount of drug taken (ng/mL) (STD)*	*Amount of drug added (ng/mL) (sample)*	*Peak area*	*Conc. found*	*SD*	*%RSD*
	50	5000	2500	53384	7500	0.62	0.20
				53384	7500		
				53384	7500		
	100	5000	5000	67489	10190	11.35	0.11
				67469	10172		
				67478	10169		
	150	5000	7500	82650	12540	23.4	0.18
				82580	12498		
				82586	12501		

Table 5: Accuracy table of ombitasvir

S. 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*
	50			11250	91146	11250	15.2	0.13
					91240	11260		
		7500	3750		91258	11280		
	100	7500	7500	15000	138752	15010	62.5	0.41
					138672	15025		
					138567	15125		
	150	7500	11250	18750	165511	18760	10.9	0.05
					165544	18776		
					165556	18755		

RTV and DRV were calculated, and the percentage RSD was also calculated.

- *Observation*

For the repeatability study, the percentage RSD for the ritonavir peak area was 0.071 and the percentage RSD for retention time was 0.12. 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 0.78. The overall results for both drugs fall under acceptable limits, indicating that this method is precise per the guidelines.

- *Acceptance requirement*

The repeatability percentage RSD cannot exceed 2.0%.

Limit of detection

The lowest concentration of analytes in a sample which is detectable but not necessary to quantified in a sample for the proposed method. In chromatograms, 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.

The detection limit is symbolized as follows (DL)

DL =3.3 σ/S

From the calibration curve slope is calculated

Limit of quantitation

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

Limit of detection (LoD) and LoQ of RTV was calculated to 300 and 850 ngm/mL; for OMB, it was 500 ng/mL of LoD and 1000 ng/mL of LoQ was found.

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 20 tablets and transferred into clean volumetric flask and diluted with methanol, sonicated for 10 minutes at ambient temperature. The sample is filtered through 0.45 micron filter and further dilution was done for analysis.

Green Assessment

Greening an analytical method as well as achieving the analytical parameters such as selectivity, specificity and limit of detection have a great challenge to analysts in developing the method under the green analytical chemistry.[13]

Green analytical procedure index

It is the most important tool widely used in assessing the greenness of the method by the analyst as it determines the greenness of all steps from sample preparation to end of analysis.[14] The evaluation criteria are measured by taking into the things like sample size, throughput, waste production, power usage, and the selection and usage of reagents, materials, and samples. The ability to discriminate between criteria and importance by giving them weights was another basis for assessment. The evaluation process was carried out using open-source, user-friendly software that produced an understandable

Table 6: Repeatability data ritonavir

Mean (Ritonavir)	*1.8538*	*8953.5*
SD	0.002	6.44
%RSD	0.12	0.071

Table 7: Repeatability data ombitasvir

Mean (Ombitasvir)	*8.089*	*6981.06*
SD	0.0063	7.44
%RSD	0.078	0.106

Table 8: Assay of tablets

Drugs name	*Percentage assay*
Ritonavir- 50 mg	99.4
Ombitasvir-12.5 mg	98.1

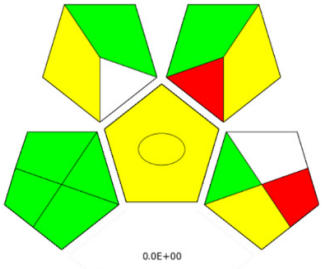


Figure 4: Complex GAPI

Figure 5: Analytical GREEnness metric approach (AGREE)

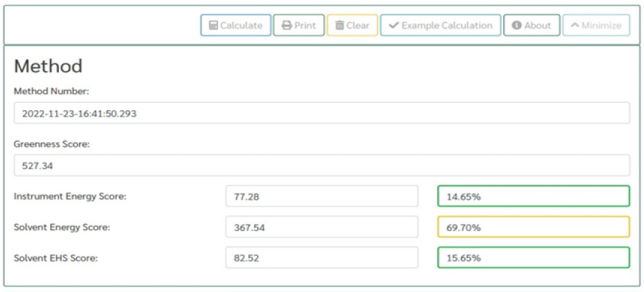


Figure 6: Analytical method greenness score (AMGS) spreadsheet calculator

pictogram with data on the overall performance and structure of threats.[15] The green analytical process index (GAPI) was first reported in 2018 and is now widely used by scientists to assess the environmental friendliness of developed techniques, which is currently fairly successful and well-established.

The GAPI metric consists of a color scale to a pictogram to categorize the level of "greenness" of each phase of an analytical steps, with two or three levels of evaluation for each stage. Reagents, practices, and equipment are assessed in GAPI. As a result, various elements are considered, including energy needs, 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.[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 reflecting zero as more hazardous and hight impact on the environment and one with the lowest impact on system and analyst.

DISCUSSION

As per the ICH guidelines, the proposed method is validated to check the purity and estimation of ritonavir and ombitasvir in pharmaceuticals bulk drugs by using eco-solvent system which is accessed for greenness by different tool and software, proven that the proposed method is green and environmentally friendly.

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 (Figure 1). 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 judge to select the mobile phase and flow rate. The mobile phase with a ratio (60:40 v/v) of methanol and isopropyl of 1-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 in different conditions and the results was found within acceptable limits. The result is shown in Table 1.

The optimization of chromatographic condition were listed in Table 2.

The standard curve (Figure 2 and 3) was plotted with different for concentration range for the ritonavir and ombitasvir from 1000 to 15000 ng/mL, and 1250 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 entire range which is selected for linearity study. The result is shown in Table 3

The specificity study examines the interference of excipients in diluent and its quantification value. According to the findings, none of the interference substances/excipients interfered with the retention time of the analytes (Table 4). As a result, the proposed method is specific as per guidelines (Table 5).

Precision parameter was examined in terms of reproducibility and repeatability were expressed in terms of %RSD were analysed by taking a more no of solution of a sample within the day (intraday) and the next three days of interday (Table 6). In each case's %RSD is examined, the values were on the low side (Table 7). This indicates that the method is precise as the percentage RSD value is in acceptable limits. For the proposed method, LoD and LoQ were determined using a signal-to-noise ratio of 2:1, indicating that the limit of detection and limit of quantification are found to be sensitive for the method. The tablet assay were done and found within the limit (Table 8)

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 color code: green, yellow and red, which indicate a low, medium, and high environmental impact. More green shade indicates high environmental safety and less risk to analysts.

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

AGREE tool

The 2nd tool is AGREE with a scale ranging from 0-1, indicating more greenness for 1 and 0 for least. By keeping the above, the proposed method its shows 0.81, which is also greener in the scale range of 0-1, indicating the method is eco-friendly and safe to analyst (Figure 5).

AMGS tool

The third tool is the analytical method greenness score (AMGS), 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 colors are intended to be an indicator highlighting the method's highest contribution to the AMGS value, indicating that the method is greener, more eco-friendly, and safer for analysts who handled routine analysis in the quality control lab (Figure 6).

CONCLUSION

A novel RP-UFLC method for ritonavir and ombitasvir was developed in this study, which employs greener chromatography and an eco-friendly solvent. This method is used to estimate ritonavir and ombitasvir because it is simple, precise, accurate, safer for analysts, and environmentally friendly. This method is appropriate for quantifying ritonavir

and ombitasvir in commercial formulations. In accordance with the GAPI, AGREE greenness, and AMGS spreadsheet metrics, the proposed method can cause less environmental impact on other organic mobile phase solvents like acetonitrile and make it safer for analysts who perform routine analysis in quality control.

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