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UFLC Method Development and Validation for Anti Retro Viral Drugs: Investigation of Greenness Assessment using Complex GAPI, AGREE and AMGS SPREADSHEET

B. Prakash Kumar

Department of pharmaceutical analysis, Sri Adichunchanagiri college of Pharmacy, Adichunchanagiri University, B G Nagar, Mandya, Karnataka- 571448, India.

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KEYWORDS

Antiviral drugs, green chromatography, UFLC., Complex GAPI,AGREE,AMGS

ABSTRACT:

The study's proposal was to establish a method development and validation by green chromatography technique for anti-viral 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 plusC18(250x4.6, 5µm) column by applying isocratic elution using the mobile phase containing methanol and isopropyl acetate in the ratio of (60:40% v/v) with 1ml/min flow rate. The separation of drugs is achieved by using greener mobile phase.

Results: The retention time of 1.854 min and 8.09 min for Ritonavir and Ombitasvir was found respectively. The regression co-efficient (R2) is 0.997 for the both drugs. Accuracy & 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 proposed method is anticipated to be eco-friendly, alternative to developed method HPLC method in regard to safe solvent, less toxic and less run time. The proposed method was adopted for the estimation of the two samples under study in their combined dosage forms.

INTRODUCTION

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

Ritonavir has a CYP3A inhibitor of HIV type 1 and protease inhibitor for HIV virus 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 maximal concentration (T_{max}) of approximately 4 to 5 hours. Steady-state exposures are achieved after approximately 12 days of dosing [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]

2019 Coronavirus Disease (COVID-19) is a new coronavirus caused by the SARS-CoV-2 virus category. It has been identified as one of the coronaviruses belonging to the Coronaviridae family for a considerable amount of time. It can cause severe fever and other respiratory disorders like pneumonia and dyspnea. One antiviral medication used to treat HIV is ritonavir; it 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]

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Green chromatography

The terms "green chemistry," "clean chemistry," "benign chemistry," and so on are the 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. Eliminating toxic, hazardous substances and replacing them with safer alternatives which benefit for analyst health and environment is the major objective of these methods. 12 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 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 green.

To assess the developed method's greenness relative to other published methods, three new techniques were adopted to measure, the Analytical Eco-Scale, Green Analytical procedure index (GAPI) and AMGS Spreadsheet [8, 9]

A tool called eco-scale is semi-quantitative in nature; it evaluates the amount of chemicals used, the risk associated with each reagent, the energy 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 LC-MS and UHPLC-MS.[8]

Moreover, a novel approach called the Green Analytical Procedure Index (GAPI), Analytical Greenness (AGREE), and National environmental method Index is used to measure the overall greenness of the method analytical process, 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 method 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 SD Fine chem ltd at Mumbai-400013. Type -I water is used in all procedure was obtained milli q of model

Instrumentation: Analysis was carried out on a prominence liquid chromatograph UFLC of Shimadzu, model LC20AD with UV- Visible detector of model SPD20A having auto sampler model SIL20AC HT with column oven temperature control. Lab solution software is used for data processing and interpretation**.**

Selection of Mobile Phase: Selection of ideal mobile phase is done by changing the composition of mobile phase in different ration, buffer composition and also by trial-and-error principle. Considering the physico-chemical properties during the preparation of mobile phase, the following criteria like, Stability, solubility, pKa value and literature review.

Mobile phase Preparation: Methanol and isopropyl acetate are prepared by adding in the ration of 60:40(*v/v*) and filtered using 0.45micron 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, 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. 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

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C-18 column is one of the most ideal and preferred for method development and validation.

Standard Solutions Preparation: Stock solution of Ritonavir and Ombitasvir were prepared in clean 100ml volumetric flask at a concentration 5mg/ml and further dilution were made to individual concentration using the mobile phase as solvent.

Ritonavir (RTV): From the above stock solution of Ritonavir, serial dilution is performed to get the concentration of 1000ng/ml to 15000ng/ml 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 concentration of 1250ng/ml to 17500ng/ml with mobile phase. A serial dilution was done to get the concentration curve which has the acceptance value

RESULTS

Reverse Phase ultra–Performance Liquid Chromatography: In the RP-UFLC method, 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. Flow rate and mobile phase selection were selected based on peak parameters like height, capacity, theoretical plates, tailing or factor, run time, and resolution.

Method Validation: -

Validation of the proposed method was done by the ICH guidelines.

Specificity: For the prosed method the specificity showed good separation of Ritonavir and Ombitasvir without any additional peaks which indicates method is specific to drug analytes. All the chromogram were analysed and found to be free from interference with drug substances**.**

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Calibration Curve and Linearity of Ritonavir and Ombitasvir: To plot the calibration curve and evaluate the correlation coefficient, the linearity of ritonavir was examined in the concentration range of (1000 ng/mL to 15000 ng/mL) and Ombitasvir (1250 ng/mL to 17500 ng/mL). The correlation coefficient (r2) was consistently higher than 0.997 for all calibration curves.

Observation:

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

Accuracy

Accuracy of Ritonavir: The accuracy of the proposed method was determined by the standard addition method. A known quantity of standard solution has been spiked into the sample solutions previously analyzed at three different levels (50%, 100%, and 150%). The experiment was carried out in triplicate, and the amount recovered for ritonavir has been calculated for three concentrations. RT and peak areas were determined, and a direct recovery study was calculated. The accuracy of the proposed method was determined by the standard addition method. A known quantity of standard solution has been spiked into the sample solutions previously analyzed at three different levels (50%, 100%, and 150%). The experiment was carried out in triplicate, and the amount recovered for ritonavir has been calculated for three concentrations. RT and peak areas were determined, and a direct recovery study was calculated.:

Accuracy of Ombitasvir: Accuracy of the proposed method was determined by the standard addition method. A known quantity of standard solution has been spiked to the sample solutions previously analysed in three different levels (50%, 100% and 150%). The experiment was carried out in triplicates and the amount recovered for ombitasvir has been calculated for three concentrations. RT and peak areas were determined and Direct recovery study was calculated:

Precision: Six different injections of the standard solution of RTV and OMB were injected simultaneously at different time intervals into a UFLC. The chromatograms obtained determine the peak area for the proposed method. The retention time and peak area of RTV and DRV were determined, and the RSD was also calculated. The result table is show in **Table 6 & Table 7.**

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Observation: The % RSD for the repeatability study was 0.12 for Retention Time and 0.071 for Peak Area of Ritonavir, respectively. As a result, the analytical method's precision was determined to be within acceptable limits. **Acceptance Criteria:** The %RSD for repeatability not more than 2.0%

Observation: The % RSD for the repeatability study was found to be 0.078 for Retention Time and 0.106 for Peak Area of Ombitasvir respectively. Therefore, the precision of the analytical method was found to be within the acceptance limits.

Acceptance Criteria: The % RSD for repeatability not more than 2.0%

DETECTION LIMIT (LOD):

The detection limit for the proposed method was carried out by considering the lowest amount of analyte in a sample which can be detected but not necessarily quantified. LOD for both drugs are performed and listed in table 9and 10.

The detection limit (DL) may be symbolised as:

DL =3.3 σ/S

Where σ = the standard deviation of the response, S is the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

QUANTITATION LIMIT (LOQ):

The lowest amount of analyte in a sample is indicates the quantification limit which can determines the low levels of drug in sample matrics and is used particularly for the estimation of impurities or degradation products**.**

The quantitation limit (QL) may be expressed as:

QL =10 σ/S

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S is estimated from the calibration curve of the analyte.

Observation:

LOD and LOQ of RTV was found to be 300ngm/ml and 850ngm/ml RTV and LOD and LOQ of OMB was found to be 500ngm/ml and 1000ngm/ml.

ASSAY(TABLET)

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

Green Assessment of proposed method.

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

It is one of the most important tools widely used in the assessment of the greenness of method by the analyst as it determines the greenness of all steps from sample preparation to end of analysis. The evaluation criteria are measured by taking into the things like sample size, throughput, waste production, power consumption, and the selection and usage of solvents, materials, and reagents. The ability to discriminate between criteria importance by giving them weights was another basis for assessment. Utilizing open-source, user-friendly software that produced an understandable pictogram with data on the overall performance and structure of threats, the evaluation process was carried out. [15] The green analytical process index (GAPI), which 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 consist of a colour scale to a pictogram to categorise the level of "greenness" of each phase of an analytical steps, 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,

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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 GREEnness calculator, a thorough, adaptable, and simple evaluation method that yields an understandable and instructive result. The evaluation criteria are converted into a single 0–1 scale and taken from the 12 principles of green analytical chemistry.

DISSCUSSION

The proposed method was validated according to ICH guidelines for the purity and estimation of **RITONAVIR**, and **OMBITASVIR**, in pharmaceuticals bulk drugs. By using various solvent system for the development of green and environmentally friendly UFLC method

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. Peak parameters (height, tailing, theoretical plates, capacity, or symmetry factor), run time, and peak resolution were used to select the mobile phase and flow rate. The mobile phase with ratio (60:40, v/v) of methanol and isopropyl at flow rate of 1.0 mL/min which indicate the method is accurate and precise.

A system suitability test was performed by taking different parameters and the method suitability test was performed to various condition and the results was found within acceptable limits of tailing factor ≤ 2.0.

The calibration curve was plotted with different range of concentration for the ritonavir and Ombitasvir from 1000ng/ml to 15000ng/ml, and 1250ng/mL to 17500ng/ml by linear least square analysis. The calibration curve of area of peak versus concentration was found to be linear and the correlation 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 draw the conclusion that this proposed method was linear throughout the range selected.

Specificity was investigated for the quantification of interference substances/excipients in diluent. According to the findings, none of the interference substances/excipients interfered with the retention time of the analytes. As a result, the developed method was specific.

The precision of the method was measured in terms of reproducibility and repeatability were expressed in term of % RSD were analyzed by taking a sufficient number of aliquots of a homogenous sample within the day (intraday) and the next three days for interday precision. Each case's %RSD was calculated, and the results were on the low side. The method was precise, as evidenced by the low RSD values.

LOD and LOQ for proposed method were determined by signal-to-noise ratio 2:1 is generally considered for acceptable which indicates that the LOD and LOQ is found to be sensitivity for the method.

The Complex GAPI consist of five pentagrams to measure and quantify the environmental effect on every step of developed method with a colour code; green, yellow and red indicating low, medium and high effect on the environment. More no of green colour shade shows high environment safety and less hazardous to analyst.

For the developed Method, its shows 8 shaded with green, 4 with yellow and 0 with Red as show in pictogram. This indicates that proposed method is very much eco-friendly and analyst safety.

The 2nd tool is AGREE with scale range from 0-1 which indicates more greenness for 1 and 0 for least. By keeping the above, for 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.

The third tool is the Analytical Method Greenness Score (AMGS), which measures the Instrument energy score, Solvent energy score, 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, 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

The method developed in this study is a novel RP-UFLC method for Ritonavir and Ombitasvir that employs a 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,

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and environmentally friendly. This method is appropriate to quantify Ritonavir and Ombitasvir in commercial formulations. This methodology is additionally feasible to quantify drugs in other physiological matrices like plasma and urine. In accordance with the GAPI, AGREE greenness, and AMGS spread sheet metrics the proposed method can reduce the environmental impact of other organic mobile phase solvents like acetonitrile and make it safer for analysts who perform routine analysis in quality control.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest.

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TABLES

Table 1: System suitability Testing for Ritonavir and Ombitasvir

SLNO	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

Table 2: - Optimization of chromatographic condition: -

Sl. 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 8.09min,
6.	Detector	UV

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7.	Column Temperature	25^0 C
8.	Wavelength	254nm
9.	Injection volume	20µl

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**

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)**	**Total amount of drug(n=3)**	**Peak area**	**Conc. found**	**SD**	**Mean conc.**	**% RSD**
1.	50	7500	3750	11250	91146	11250	15.27	11263	0.13
					91240	11260			
					91258	11280			
2.	100	7500	7500	15000	138752	15010	62.5	15053	0.41
					138672	15025			
					138567	15125			

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3.	150	7500	11250	18750	165511	18760	10.9	18763	0.05
					165544	18776			
					165556	18755			

STD- Standard, SD- Standard Deviation, RSD- Relative Standard Deviation

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**	**Mean conc.**	**% RSD**
1.	50	5000	2500	7500	53384	7500	0.62	299.72	0.20
					53384	7500			
					53384	7500			
2.	100	5000	5000	10000	67489	10190	11.35	10177	0.11
					67469	10172			
					67478	10169			
3.	150	5000	7500	12500	82650	12540	23.4	12513	0.18
					82580	12498			
					82586	12501			

STD- Standard, SD- Standard Deviation, RSD- Relative Standard Deviation

Table 6& 7: Repeatability Data Ritonavir and Ombitasvir

Mean	**1.8538**	**8953.5**
SD	**0.002**	**6.44**
%RSD	**0.12**	**0.071**
Mean	8.089	6981.06
SD	0.0063	7.44
%RSD	0.078	0.106

Table 8: Assay of Tablets

Drugs name	Percentage Assay
Ritonavir- 50mg	99.4
Ombitasvir-12.5mg	98.1

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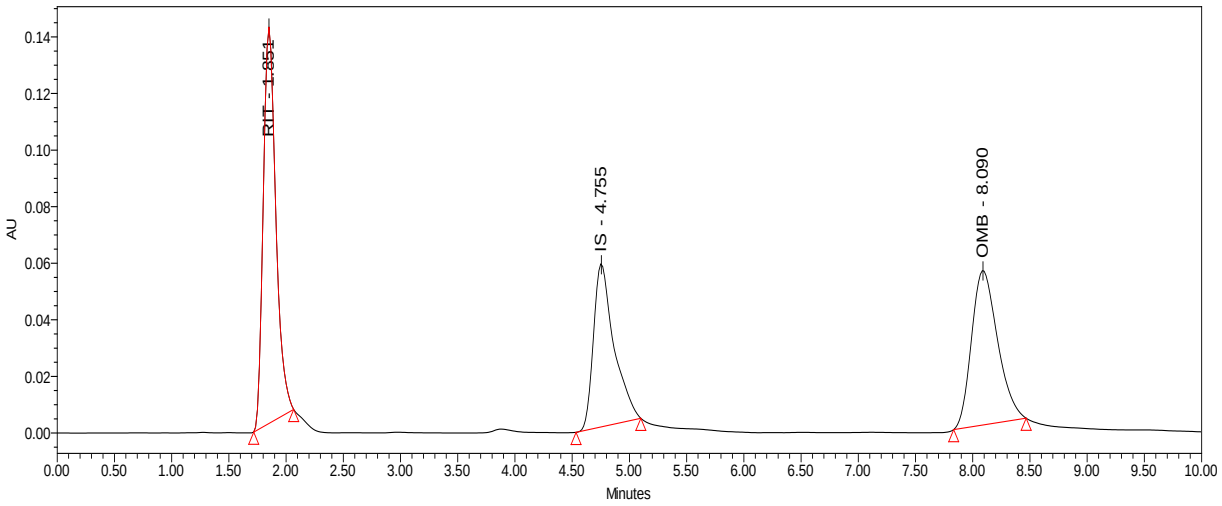

Fig 1: Standard chromatogram of Ritonavir and Ombitasvir

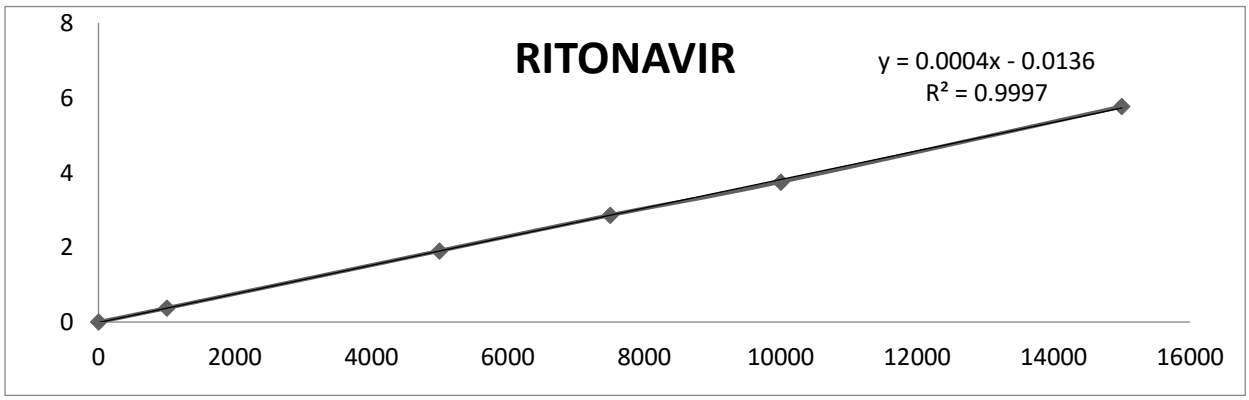

Fig 2: Standard curve data for Ritonavir

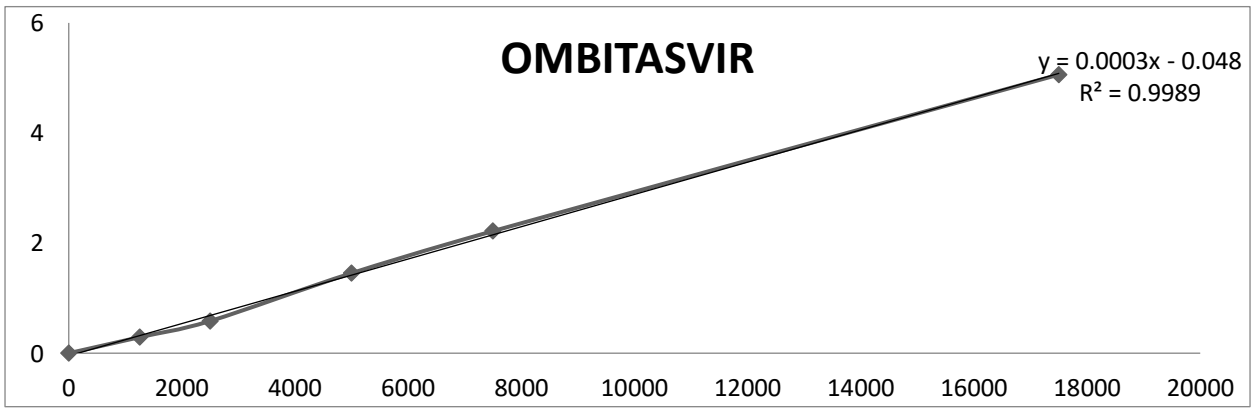

Fig 3: Standard curve data for Ombitasvir

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Fig 4: -AGREE (Analytical GREEnness Metric Approach)

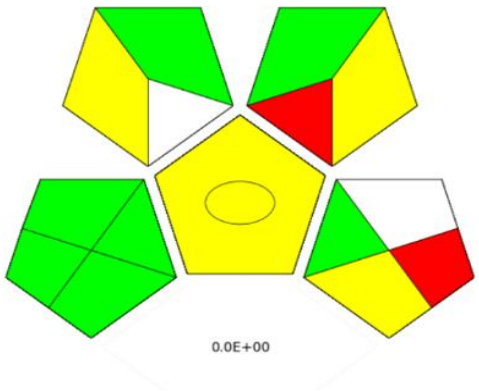

Fig 5: Complex GAPI (Green Analytical Procedure Index)

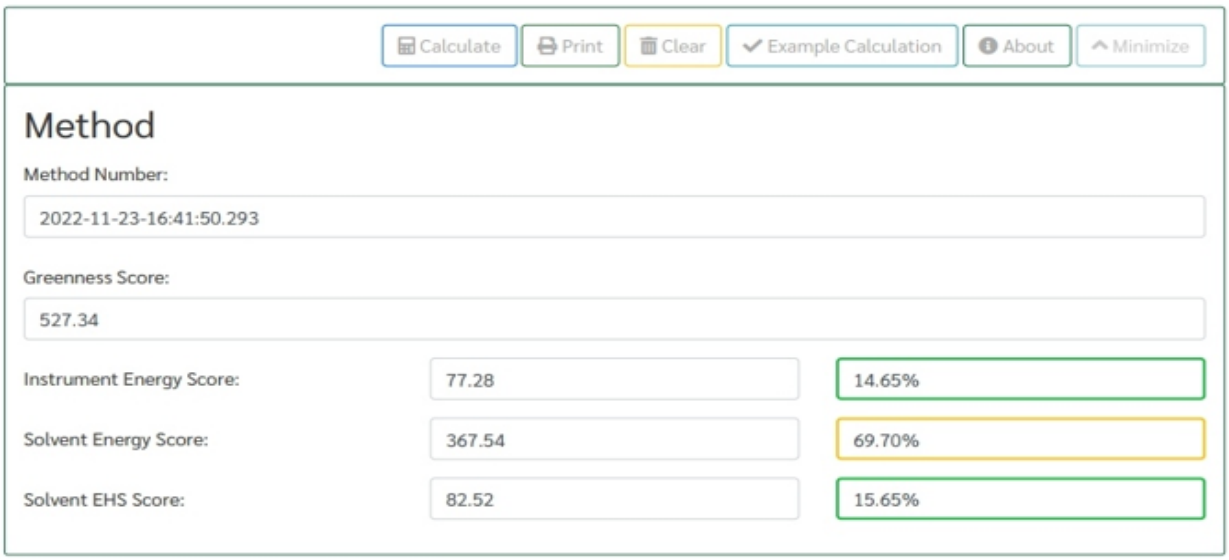

Fig 6: AMGS (Analytical Method Greeness Score) Spreadsheet calculator