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Validated green spectroscopic manipulation of area under the curve (AUC) for estimation of Simvastatin: Application to nano-structured lipid carriers and niosomal systems

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ABSTRACT: This work introduced a green spectrophotometric method based on the manipulation of the area under the curve named Area under the curve correction method (AUCCM). The method was applied for the characterization and evaluation the in-vitro release of two nanocarrier systems namely, nano-structured lipid carrier (NLC) and niosomal system of Simvastatin (SIM), through measuring free SIM in presence of the possible excipients/interferents (oleic acid and cholesterol) in a challenging matrix. The estimation of SIM was calculated using the area under the curve (AUC) in the wavelength range of (234.5 - 245.5) nm which includes the λmax of SIM in the concentration range of (1-35 µg/mL). The method was validated in compliance with ICH guidelines. The greenness of the developed analytical method will be assessed and compared to other reported methods using the greenness assessment tools known as: National Environmental Methods Index (NEMI), Green Analytical Procedure Index (GAPI) and Eco-Scale. The method proved to be the best regrading greenness and and was successfully applied for the in-vitro release study of SIM from the two nano carrier systems.

KEYWORDS: Niosome; Nano-structured lipid carrier; Area under the curve; Green Analytical Procedure Index; Eco-scale.

1. INTRODUCTION

Simvastatin (SIM) (1S,3R,7S,8S,8aR)-8-[2-[(2R,4R)-4-hydroxy-6-oxotetrahydro-2H-pyran-2-yl] ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate. SIM is a white crystalline powder which is practically insoluble in water but freely soluble in alcohol. It is absorbed from the gastrointestinal tract and then hydrolyzed to its active β-hydroxy acid form. SIM undergoes extensive first-pass metabolism in its primary site of action, the liver, which leads to very poor oral bioavailability (less than 5% of the oral dose reaches the circulation as active metabolites). About 95 % of SIM and its active metabolite are bounded to plasma proteins. The half-life of the active metabolite is around 2 hours [1].

Simvastatin (SIM) acts as a competitive inhibitor to HMG Co-A reductase enzyme leading to reduced cholesterol and lower levels of intracellular cholesterol [2]. Through the last decades, researches described pleiotropic effects of simvastatin including the improvement of endothelial dysfunction and increasing myocardial perfusion by increasing nitric oxide bioavailability through downregulation of endothelial nitric oxide synthase (eNOS) [3]. Other reported effects were antioxidant effects, anti-inflammatory properties, stabilization of atherosclerotic plaques, immunosuppressive activity and inhibition of cardiac hypertrophy [4]. SIM was described for the treatment of indications rather than hyperlipidemia such as rheumatoid arthritis [5]. Recent studies showed that SIM has an important role in cell proliferation, so it was described for chronic

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wound healing [6] and bone regeneration [7]. The most commonly-known dosage form of SIM in the market is the film-coated tablets used as a controlled release lipid-regulating drug [8]. Other formulations were described such as self-emulsifying drug delivery systems [9,10], nanoparticles [7,11-13], buccal film of SIM [14] floating tablets [15] and dry powder inhaler [16].

Literature revealed reversed phase high performance liquid chromatography (RP-HPLC) methods for the analysis of SIM in different formulations in presence of co-formulated drugs, components and/or excipients [17-21]. In addition to HPLC methods, several spectroscopic approaches were reported for the analysis of SIM including colorimetric methods [22-24] , ultraviolet (UV) spectrophotometric methods [25-28] and chemometric methods [29,30].

An important concept that has emerged in last few decades is "Green chemistry" also known as sustainable chemistry, which is focused on the design of products and processes that minimize or eliminate the generation and use of hazardous material. The green chemistry is explained through twelve principles [31] which includes "Less Hazardous Chemical Syntheses", "Designing Safer Chemicals" and "Safer Solvents and Auxiliaries". Therefore, there is a demand for manufacturing pharmaceutical formulations using substances of natural origin that possess no toxicity to human health and the environment; and develop an analytical approach for its quantitation with green solvents and minimum waste generation.

Although HPLC methods are rapid and reproducible in comparison to other analytical methods, but it involves the use of organic solvents such as (methanol and acetonitrile) and hyphenated instrument which may affect the greenness of the analytical method. Spectroscopic approaches are simple greener alternatives to chromatographic methods involving the use of green solvents with lower voltage, waste and cost. On the other hand, chromatographic methods transcend in the resolution and quantitation of active ingredients in different matrix, while interference of other components or excipients is not easily resolved using spectroscopic approaches as in HPLC. So, upon performing quantitation of multi-component matrix using spectroscopic approach, certain manipulations should be applied [32-34].

The aim of this work is to develop a green spectroscopic method for the characterization and evaluation of two nanocarrier systems, nano-structured lipid carrier NLC and a niosomal SIM system, through measuring free SIM in presence of the possible excipients/interferents such as cholesterol and oleic acid. The conventional spectrophotometric methods were not applied successfully in estimating SIM in presence of the excipients in its pharmaceutical formulation due to interfering signals. Therefore, the green spectroscopic approach "Area under the curve correction method (*AUCCM*)" utilizes the area under the curve (AUC) for the successful quantitation of SIM in this challenging matrix instead of hyphenated high-cost chromatographic conditions. The greenness of the developed analytical method will be assessed using the greenness assessment tools known as: National Environmental Methods Index (NEMI), Green Analytical Procedure Index (GAPI) and Eco-Scale.

1.1. Theory of Area under the curve correction method (AUCCM)

The well-known spectrophotometric method area under curve (AUC) calculate the concentration of the component of interest by measuring the area of its curve in zero order [35,36] or ratio absorption spectrum [37]. A simple modification was introduced to AUC method [34] using absorption correction method for AUC with no need for Cramer's rule [38]. The AUC values are used instead of absorbance values for the same component, which increases sensitivity even in lower concentrations.

For a binary mixture (X and Y components) with partial overlapped spectra at the region of (λ_1 - λ_2), while X shows no interference with Y at the extended wavelength region (λ_3 - λ_4). The component "Y" is determined though the AUC in (λ_3 - λ_4) directly due to lack of absorption of component "X" at the extended region, while the AUC of X at (λ_1 - λ_2) can be calculated using the following equation:

AUC of X at (λ_1 - λ_2) = AUC at (λ_1 - λ_2) of X+Y - $\frac{AUC(1-2)}{AUC(3-4)}$ × AUC at (λ_3 - λ_4) of X+Y

Where; $\frac{AUC(1-2)}{AUC(3-4)}$ is AUC factor of pure Y [AUC at (λ_1 - λ_2) / AUC at (λ_3 - λ_4)], AUC at (λ_3 - λ_4) of X+Y = AUC at (λ_3 - λ_4) of Y only.

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2. RESULTS AND DISCUSSION

2.1. Spectral characteristics and profiles

By proposing the new "area under the curve correction" method (AUCCM), area under curve (AUC) and absorption correction methods have been merged [44]. Maximum sensitivity and selectivity were obtained due to measuring area of component in a wide range of wavelengths instead of a single wavelength which may be subjected to shifting. This method prevails over derivative-manipulating methods in terms of sensitivity by eliminating instrumental noise.

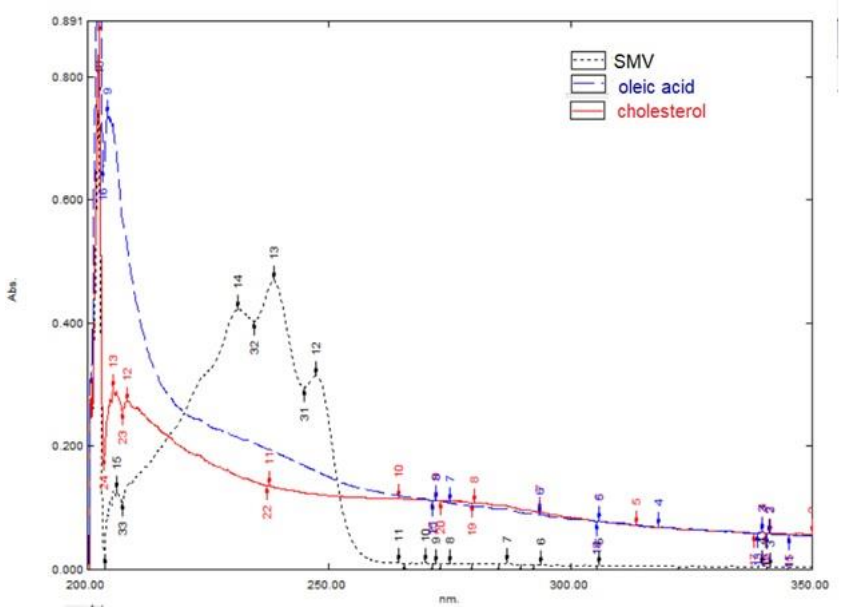

Figure 1. The zero order absorption spectra of SMV, oleic acid and cholesterol in phosphate buffer (pH 7.4)

For preparations containing *SIM*, oleic acid and/or cholesterol, direct measurement of *SIM* can't be performed due to interference of the zero order D_0 spectra of oleic acid and/or cholesterol with the D_0 spectrum of *SIM* in the wavelength range 234.5 - 245.5 (nm), which includes the λ_{max} of SIM. On the other hand, pure oleic acid and cholesterol exhibits absorbance with no interference of *SIM* in the wavelength range of (280 - 305) nm as shown in Fig. 1. Therefore, The AUC factors for pure oleic acid and cholesterol were calculated as $\frac{AUC(234.5-245.5)}{AUC(280-305)}$ which is corresponding to the ratio of the AUC at the interference wavelength range 234.5 - 245.5 (nm) divided by the AUC at the non-interference wavelength range 280 - 305 (nm). The AUC factor of pure oleic acid or cholesterol was calculated to be equal to 0.81 and 0.62, respectively. No spectral interference was observed for SDS, stearic acid, Tween 80 or Span 60 in PBS solutions.

Accordingly, *SIM* concentration was estimated using the "Area under the curve correction method (*AUCCM*)" to eliminate the interference oleic acid and/or cholesterol as shown in Fig. 2 and 3. The AUC corresponding to *SIM* in the region of at 234.5 - 245.5 (nm) (λ_1– λ_2) can be calculated using the AUC factor as follow:

AUC of SIM (234.5 - 245.5) = AUC (234.5 - 245.5) of mixture - [$\frac{AUC(234.5-245.5)}{AUC(280-305)}$ × AUC (280 - 305) of mixture]

Where; $\frac{AUC(234.5-245.5)}{AUC(280-305)}$ is AUC factor of pure oleic acid or cholesterol and the mixture corresponds to the preparation containing (*SIM* + oleic acid) or (*SIM* + cholesterol).

The concentration of SIM was calculated by the calibration curve relating the AUC at the wavelength range (234.5 - 245.5 nm) with its concentration after eliminating the interference of oleic acid or cholesterol.

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2.2. Validation sheet

Method validation was performed according to ICH guidelines [45] using both solvents.

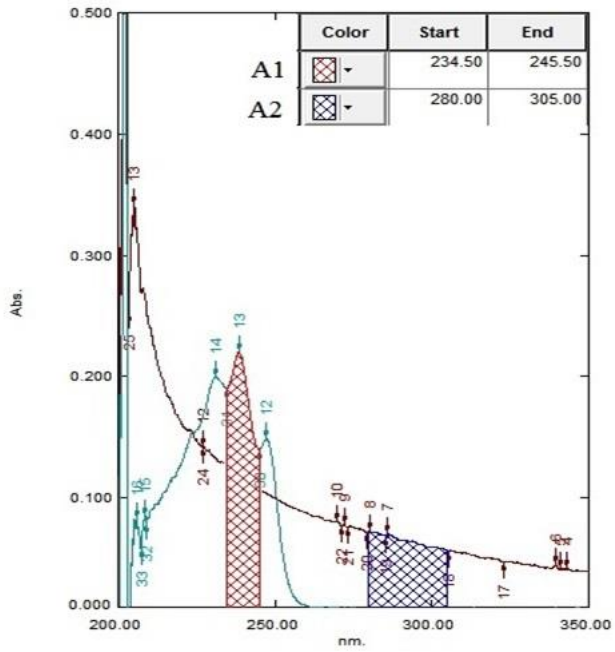

Figure 2. Zero order absorption spectra of SMV and oleic acid showing the AUC regions (234.5-245.5 nm) and (280-305 nm)

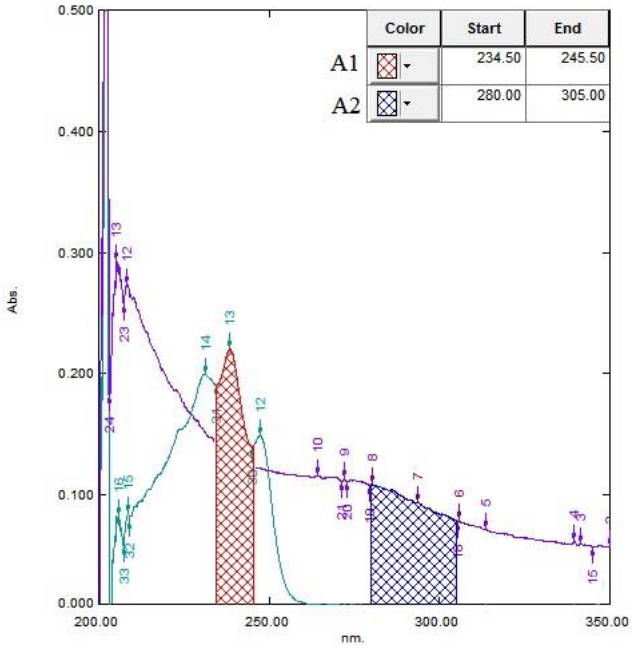

Figure 3. Zero order absorption spectra of SMV and Cholesterol showing the AUC regions (234.5-245.5 nm) and (280-305 nm)

2.2.1. Range and linearity

The linearity of the proposed method was evaluated by carrying out calibration curve on three successive days. The calibration curve was constructed within concentration ranges that were selected on the basis of the anticipated drug concentration during the assay of the formulations, as shown in **Supplementary material Fig.1SM**. The concentration for *SIM* was found to be linear in relation to area under the curve at 234.5 - 245.5 (nm), in the range of (1-35 µg/mL). Linearity was assessed by calculating the correlation coefficient "r". The

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concentration range, calibration equations and other statistical parameters for the proposed method were listed in Table 1.

2.2.2. Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated respectively, for SIM using the proposed method with a ratio of 3.3 and 10 standard deviations of the blank and the slope of the calibration line, as listed Table 1.

LOD = 3.3 * SD of intercept / slope of calibration line

LOQ = 10 * SD of intercept / slope of calibration line

2.2.3. Accuracy

The procedure under "*Area under the curve measurements*" were repeated three times for the five determination of different concentrations of SIM within the linearity range. The accuracy was calculated and expressed as mean percentage recoveries and standard deviation (SD) as shown in Table 1.

Table 1. Validation sheet and regression parameters for the proposed method for the determination of SMV.

Method	**AUC (234.5 - 245.5 nm)**	
Calibration range (µg/mL)	1-35	
LOD (µg/mL)	0.24	
LOQ (µg/mL)	0.73	
Slope	0.4493	
Intercept	- 0.1699	
Correlation coefficient (r)	0.9994	
r^2	0.9996	
Mean % [a]	100.08	
SD [a]	± 1.32	
Accuracy [b] (Mean± SD)	99.77 ±1.33	
Robustness [a]	100.25 ±1.21	
Selectivity	100.99 ±1.33 [c]	99.86 ±0.96 [d]

[a] Average of three experiments.
[b] Average of five determination.
[C] for NLC combinations (SIM + oleic acid)
[d] for Niosomal combinations (SIM + cholesterol)

2.2.4. Precision

The intra-day precision (repeatability) of the proposed methods were determined by the analysis of three different concentrations of *SIM*, within the linearity range, by three replicate analyses of three pure samples on a single day. For the inter-day precision, the previous procedure was performed on three consecutive days. The results expressed as relative standard deviations (%RSD) were illustrated in Table 2.

Table 2. The intra-day precision (repeatability) and inter-day precision of the proposed method.

SMV concentration (µg/mL)	**intra-day precision**	**inter-day precision**
5	100.25	101.25
	101.25	100.63
	100.98	98.63
25	99.58	99.62
	100.21	99.10
	99.63	101.63
45	100.54	102.32
	101.23	101.96
	99.85	100.58
% RSD	0.649	1.284

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2.2.5. Selectivity

Selectivity was ascertained by analyzing different synthetic mixtures containing *SIM*, oleic acid and/or cholesterol in different ratios within the linearity range. Satisfactory results were shown in Table 1.

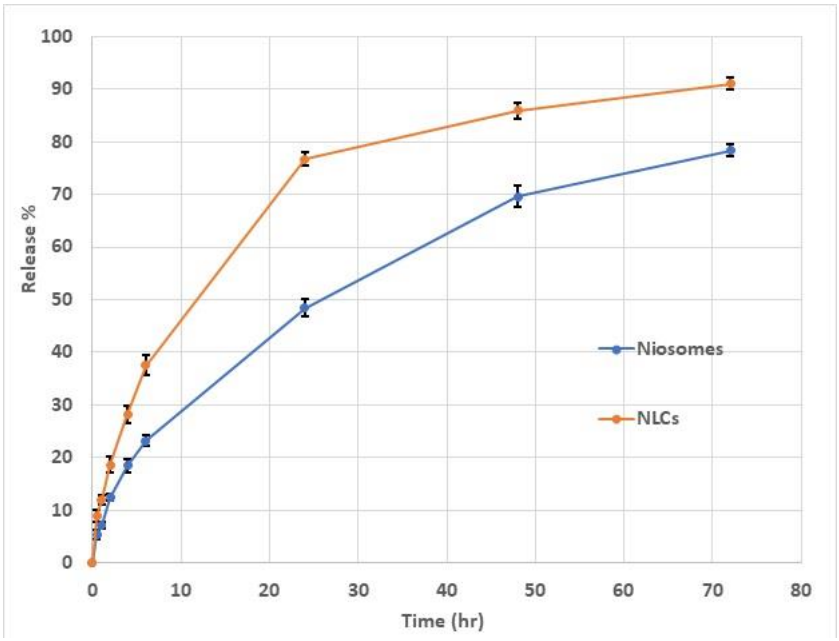

Figure 4. *In vitro* release of SIM from the prepared nanocarriers using the developed method.

2.2.6. Robustness

Testing robustness involved performing the spectral measurements at ±2 nm for three different concentrations of SIM. The robustness was calculated as mean percentage recoveries and standard deviation as shown in Table 1. No significant changes were observed, so the method proved to be robust.

2.3. Application to *In vitro* release study

The concentration of pure SIM of the withdrawn samples at the selected time intervals (0.5, 1, 2, 4, 6, 8, 24, 48, and 72 h) were calculated after elimination of the excipients (oleic acid and cholesterol) as shown in Table 3. The release percentage and standard deviations of the triplicate samples were calculated as a ratio from the initial concentration (equivalent to 35 µg/mL) and was plotted as a function of time as shown in Fig. 4. Niosomal systems showed higher release% from the begining which extended to the end of the release study.

Table 3. *In vitro* study of SIM release from NLC and Niosomal systems.

Time (hr)	NLCs			Niosomes		
	SIM conc	Release %	± SD	SIM conc	Release %	± SD
0	0	0	0	0	0	0
0.5	3.12	8.90	1.12	1.91	5.47	0.88
1	4.22	12.05	0.83	2.52	7.19	0.73
2	6.52	18.62	1.55	4.37	12.50	0.75
4	9.92	28.33	1.65	6.47	18.49	1.30
6	13.13	37.52	1.90	8.13	23.22	1.02
24	26.83	76.67	1.30	16.97	48.48	1.59
48	30.08	85.93	1.53	24.37	69.62	1.97
72	31.87	91.05	1.11	27.43	78.38	1.10

2.4. Greenness assessment and Comparison to other reported methods

Green chemistry aims for developing eco-friendly procedures for developing and analyzing pharmaceuticals with its highest priority for researchers and analysts. That's why greenness assessment represents an important criterion nowadays in the development and optimization of any proposed method. Gałuszka et al. [46] introduced 12 main principles affecting green chemistry. Consequently, four tools were presented to assess greenness base

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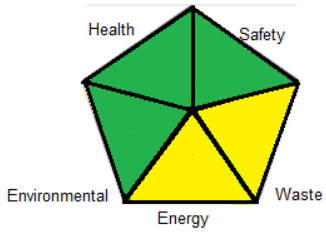
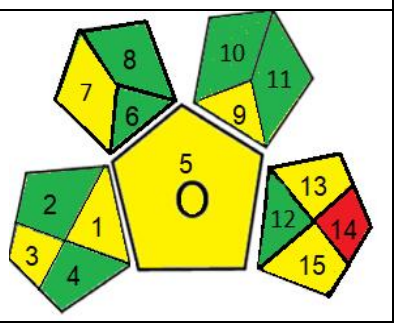
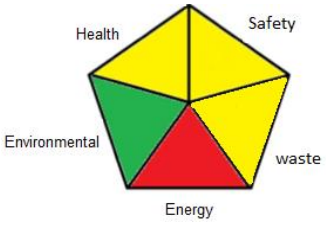
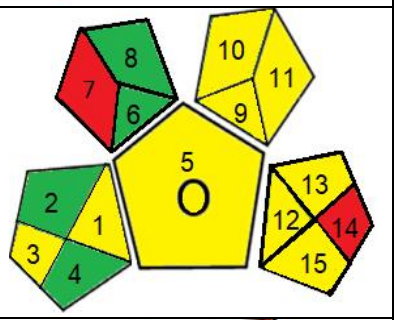
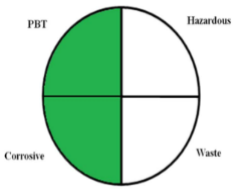
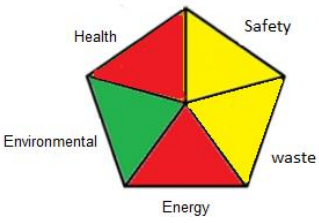
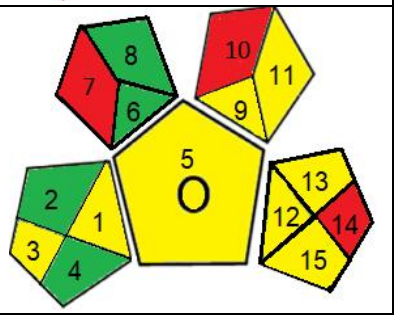
on those 12 principles which are: National Environmental Methods Index (NEMI), Assessment of Green Profile (AGP) and Green Analytical Procedure Index (GAPI) and Eco-Scale.
No spectrophotometric methods were reported for the analysis of SIM in presence the non-active ingredients of interest (Tween 80, stearic acid, oleic acid, cholesterol and Span 60). Therefore, the proposed AUCCM method was compared to two reported chromatographic method in terms of greenness assesment tools. The first method was reported for the analysis of NLC of SIM using a stationary phase C_{18} coulumn and a mobile phase at a mixture of acetonitrile and 0.1% phosphoric acid (85:15, v/v) at pH 4 at flow rate of 1.5 mL/min and UV detection at 238 nm [47]. The second method was reported for the analysis of niosomal system of SIM using a stationary phase C_{18} coulumn and a mobile phase of acetonitrile and formic acid 3 mM (51:49 v/v) at flow rate of 1 mL/min and UV detection at 238 nm [48].

Table 4. Green assessments and comparison using NEMI, AGP and GAPI tools

Greenness tool	NEMI	AGP	GAPI
Proposed method for NLC and niosome			
Reported method for NLC [47]			
Reported method for niosome [48]			

2.4.1. National Environmental Methods Index (NEMI)

National Environmental Methods Index (NEMI) was the first simplest qualitative tool for greenness assessment [49], presented by a pictogram consisting of four quadrants either shaded in green or left blank. The quadrant is green shaded if:

a) All reagents are not defined as PBT reagents (persistent, bio-accumulative, and toxic) by the Environment Protection Agency's Toxic Release Inventory (EPA-TRI) [50].

b) None of the chemicals applied in the procedure is listed on D, F, I or C hazardous wastes EPA list [51].

c) The pH lies between 2-12.

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d) The waste produced during the procedure is less than 50g.

By assessing the proposed method via NEMI, it was found that proposed method has a green profile with three green-shaded quadrants (a-c) as shown in Table 4. Quadrant "d" was left blank as the waste generated from the method will exceed 50g. The same assessment was observed for the reported method of NLC [47]. On the other hand, the reported method for niosomes [48] used 49% formic acid as a mobile phase; and that's why quadrant "c" was also left blank.

2.4.2. Assessment of Green Profile (AGP)

This tool is a semi-quantitative tool presented by a pentagram divided into five risk potentials: health, safety, environmental, waste, and energy [52]. Each potential can be shaded by either green, yellow or red. The health and safety potentials are shaded according to National Fire Protection Association (NFPA) codes and standards [53]. Environment and waste pentagrams are shaded according to its yield (less than 50 g, 50-250 g and more than 250 g). Energy pentagram is shaded according to the solvent evaporation produced by the instrumentation.

By assessing the proposed method as shown in Table 4, Three potentials were green shaded, as the solvents used has NFPA health and safety scores (green shading) and low environmental impact low of phosphate buffer solution (PBS), but the two potentials: energy consumption and waste produced were yellow shaded. The greenness assessment of both reported methods [47, 48] showed two yellow potentials (safety and waste) and one red potential (energy). The health potential is red shaded in the reported method for niosomes [48] because of the use of formic acid, while for the reported method of NLC [47] it was yellow shaded because of the use of acetonitrile.

2.4.3. Green Analytical Procedure Index (GAPI)

This is a semi-quantitative tool of greenness assessment introduced by Wasylka [54] assessing 15 analytical aspects divided into five pentagrams subdivided into smaller sections which are green-yellow to red shaded according to impact of each section from low to high as shown in **Supplementary material Table.1SM.**

The proposed AUCCM method showed GAPI pentagrams with seven green-shaded aspects related to sample preservation, storage, preparation and treatment and safety and a yellow solvent pictogram (no.7) due to the use of green solvent. The waste aspect was red-shaded (> 10mL), as shown in Table 4. The greenness assessment of both reported methods [47, 48] showed a red solvent pictogram (no.7) due to the use of non-green solvents, and yellow pictograms (no. 11 & 12) due to safety and energy consumption. The health pictogram (no.10) is red shaded in the reported method for niosomes [48] because of formic acid, but it is found to be yellow for the reported method of NLC [47] because of acetonitrile.

2.4.4. Analytical Eco-Scale

The analytical Eco-Scale [55] is an excellent quantitative tool to verify greenness of an analytical method. Analytical Eco-Scale is calculated by calculating penalty points for every factor in the analytical procedure that affects perfect green analysis. The sum of penalty points is subtracted from 100 to calculate the eco-scale calculation as shown in the following equation:

$$\textit{Analytical Eco-Scale} = 100 - (\textit{Total penalty points})$$

A green analysis is considered ideal if the Eco-Scale is equal to100, excellent if >75, acceptable if >50, and inadequate if <50. By calculating the Eco-Scale for the proposed method as shown in Table 5, it was found that the eco-scale was equal to be 90 points, which is considered a relatively high score due to the use of green solvents and non-hazardous chemicals only but with high amount of waste due to preparation of several working solutions.

The reported methods [47, 48] showed lower eco-scales, due to the use of non-green solvents (acetonitrile, phosphoric and formic acids), high flowrates and retention times which in turn increased amount of waste relative to other chromatographic methods. This led to increasing the calculated penalty points and the eco-

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scale was calculated to be 83 and 81, respectively. The Eco-scale was found to be the most sensitive and accurate tool which could analyze the greenness differences between the proposed and the reported methods precisely.

Table 5. Eco-Scale and Penalty points (PPs) for estimating the greenness of the proposed and reported methods.

	Penalty points (PPs)		
Reagents	**Proposed AUCCM**	**Reported NLC method [47]**	**Reported niosome method [48]**
Acetonitrile	0	8	8
Formic acid	0	0	6
Phosphoric acid	0	4	0
Disodium hydrogen phosphate	2	0	0
Sodium dihydrogen phosphate	2	0	0
Instrument Energy	1	2	2
Occupational hazard	0	0	0
Waste	5	3	3
Total PPs	∑ 8	∑ 17	∑ 19
Analytical Eco Scale total score	90	83	81

3. CONCLUSION

This work discussed the development and validation of the "Area under the curve correction method" (AUCCM) which is a spectrophotometric method based on manipulating the area under the curve AUC. The method is based on calculating the AUC factor for interferents or excipients to eliminate their AUC from the mixture's spectra. The method was applied for the characterization and evaluation of two nanocarrier systems namely, nano-structured lipid carriers (NLC) and Simvastatin niosomal system, through measuring free SIM in presence of the possible excipients/interferents (oleic acid and cholesterol) in a challenging matrix. The estimation of SIM was calculated using the AUC in the wavelength range of (234.5 - 245.5) by calculating the AUC factors of oleic acid and cholesterol. The method was validated in compliance with ICH guidelines and was successfully applied for the in-vitro release study of the two SIM formulations. The method was assessed to be a greener method than the reported methods, which eliminates the generation and use of hazardous material and minimizes waste using the tools: NEMI, GAPI and Eco-Scale.

4. MATERIALS AND METHODS

4.1. Instrument and software

UV-Visible double beam spectrophotometer (Shimadzu - UV 1800 - Japan), with matched 1 cm quartz cells, equipped with system software of Shimadzu UV-Probe 2.32. Scans were carried at (200-400 nm) using 0.5 nm interval.

4.2. Chemicals and standard solutions

Pure samples of simvastatin (*SIM*), oleic acid and cholesterol were supplied by local pharmaceutical companies. The purity was tested by the official methods [39] which was found to be 99.88 ±0.15 for SIM. Ethanol (s.d.fine-chem limited- Mumbai), disodium hydrogen phosphate, sodium dihydrogen phosphate, phosphoric acid, oleic acid, cholesterol and sodium dodecyl sulphate (SDS) (Adwic – Egypt). All chemicals used were of analytical grades. Distilled water was used.

Stock solutions of *SIM*, oleic acid and cholesterol were prepared in concentration 1 mg/mL using phosphate-buffered saline (PBS, pH 7.4) as solvents. Working solutions were freshly prepared by dilution from the stock solutions using phosphate buffer (pH 7.4) to obtain a concentration 50 µg/mL for SIM, oleic acid and cholesterol. Different aliquots were drawn from the working solutions and diluted with the same solvent to prepare the following concentration ranges of (1-35 µg/mL) of SIM, (5-30 µg/mL) of oleic acid and cholesterol.

4.3. Area under the curve measurements

The area under the curves (AUC) were measured for the zero order absorption spectra D_0 of *SIM* in the wavelength range 234.5 - 245.5 (nm), where calibration curve in phosphate-buffered saline (PBS, pH 7.4) was

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constructed for *SIM* by plotting the AUC values versus their corresponding concentrations at the mentioned wavelength range and the regression equation was computed. The area under curves of the obtained zero order absorption spectra D_0 of *SIM*, oleic acid and cholesterol were recorded in the wavelength ranges 234.5 - 245.5 (nm) (λ_1– λ_2) and 280-305 (nm) (λ_3– λ_4). The AUC factors $\frac{AUC(234.5-245.5)}{AUC(280-305)}$ for pure oleic acid and cholesterol were calculated, which is corresponding to the ratio of [AUC at 234.5 - 245.5 (nm)/ AUC at 280-305 (nm)].

4.4. Preparation of SIM NLC and Niosomal systems

Both nanocarrier systems contain SIM with the addition of certain excipients required for their preparations. For the preparation of nano-structured lipid carrier NLC, the non-active ingredients were Tween 80, stearic and oleic acids. While for the preparation of the niosomal system, the excipients were cholesterol and Span 60. The detailed procedure of preparations of both systems are described in an ongoing manuscript under publication.

4.5. Applying AUCCM in vitro release of SIM from the prepared nanocarriers

In vitro release of SIM from the optimized NLC and Niosomal systems was studied under sink conditions using the dialysis method [40]. Samples of each system (equivalent to 3.5 mg of the drug) were packed into the dialysis bag and immersed in 100 mL of phosphate-buffered saline (PBS, pH 7.4) with 0.5% SDS in a bottle. The bottles were placed in a thermostatic shaker at 37 °C and 100 rpm. Two-milliliter aliquots were withdrawn at pre-determined time intervals (0.5, 1, 2, 4, 6, 8, 24, 48, and 72 h) as triplicates and replaced immediately with equal volumes of fresh PBS to maintain the same volume. The withdrawn aliquot at each time interval was analyzed for SIM spectrophotometrically at a wavelength range of 200-400 nm. The cumulative amount of drug release was calculated as a function of time [41-43].

The measured AUC of each sample is measured at the wavelength ranges: (234.5 - 245.5 nm) and (280-305 nm) against 0.5% SDS as a blank. For NLC samples, the AUC at (280-305 nm) is multiplied by the AUC factor $\frac{AUC(234.5-245.5)}{AUC(280-305)}$ of oleic acid in order to obtain the AUC corresponding to oleic acid in the (234.5 - 245.5 nm), then this calculated AUC is subtracted from the total AUC of the sample at the same wavelength range to obtain the corresponding AUC of pure SIM in the range (234.5 - 245.5 nm). For the niosomal SIM system, the same procedure was adopted using the AUC factor $\frac{AUC(234.5-245.5)}{AUC(280-305)}$ of cholesterol. The concentration of SIM was calculated at each time interval and the release % was plotted against time.

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