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Green and white-assessed validated chromatographic methods for Ondansetron purity testing in its pharmaceutical formulations; *in silico* toxicity profiling of impurities

Christine M. El-Maraghy [a], Mai S. Nour [b], Heba T. ELbalkiny [a,*]

[a] *Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 11787 6th October City, Egypt*
[b] *Pharmaceutical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 11787 6th October City, Egypt*

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ABSTRACT

Drug impurities are seen as a crucial threat to drug safety, specifically when dealing with mutagenic/ toxic impurities. Here, we present LC-MS/MS and HPTLC-densitometric methods for simultaneous quantification of Ondansetron and its four official impurities. For the LC-MS/MS, the isocratic elution was applied using methanol and water containing 0.1 % formic acid in a ratio (70:30 v/v) at a flow rate of 1 mL/min, a stationary phase C_{18} column (4.6 × 50 mm, 5 μm) and mass detection using the MRM mode. For the HPTLC-densitometric method, the mobile phase consists of ethyl acetate: methanol in a ratio (6:4 v/v), and the UV detection was at 216 nm. The developed methods have been validated per ICH recommendations and then evaluated using five tools for whiteness and greenness assessment, offering promising results in comparison to reported chromatographic methods. Additionally, the toxicity profile of the impurities was expected by the online software; PreADMET and pkCSM. The developed methods are recommended for quality control due to their high analytical performance as well as their sustainability, simplicity, and cost-effectiveness, which improves the surveillance capability.

1. Introduction

The presence of impurities in drug products, even in minute amounts, may affect their safety and efficacy. Many impurities were proved to be toxic and/or mutagenic. Consequently, their detection and quantitation are mandatory. The impurities may be byproducts or intermediates arising during the manufacturing process of active pharmaceutical ingredients (API). The impurities have a closely related structure to the API, making their detection and analysis challenging. As per International Council for Harmonization (ICH) guidelines [1], more than 0.1 % of Impurities should be identified and characterized.

Ondansetron (OND) is a commonly used anti-emetic drug, especially for pregnant women, or co-administered with chemotherapy to prevent induced nausea and vomiting [2]. OND is chemically (RS)-9- methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-2,3-dihydro-1H-carbazol-4(9H)-one [3], it is a selective 5-HT3 receptor antagonist which blocks the serotonin action [4]. The monograph of OND in British Pharmacopoeia (BP) and United States Pharmacopoeia (USP) listed eight (A-H) and four (A-D) impurities respectively [5,6]. Two of the mentioned impurities were reported to be mutagenic; Impurity E (imidazole) and impurity F (2-methyl-imidazole) [7]. Impurity D is toxic if its level exceeds 0.1 % and it is the product of the alkaline degradation of OND using 1 M NaOH for 30 h at 80 °C [8]. For impurity G, is also a metabolite, but its toxicity profile data is not available. Thus, a further study to evaluate the toxicity properties of OND and impurities is needed. The toxicity profiling of impurities is laborious, time-consuming, costly, and of ethical concerns. For these reasons, computational tools were raised in order to predict and estimate the toxicity profile of drugs while reducing the cost and time. Herein, Open-access database servers such as PreADMET and pkCSM were employed to determine in silico toxicity profiling, which are time and cost-effective.

Recent awareness of environmental pollution and contamination of natural sources, due to incorrect disposal of harmful chemicals and solvents, has made green chemistry principles implementation in analytical methods essential. In this regard, the goal of the current study is to use a greener chromatographic approach to determine the levels of OND and its impurities. The three reported chromatographic methods coupled to a UV detector for OND analysis with its impurities did not concede with the green principles as the run time of the three methods was more than 18 min which led to an increase in the amount of solvent

* Corresponding author.
E-mail address: htarek@msa.edu.eg (H.T. ELbalkiny).

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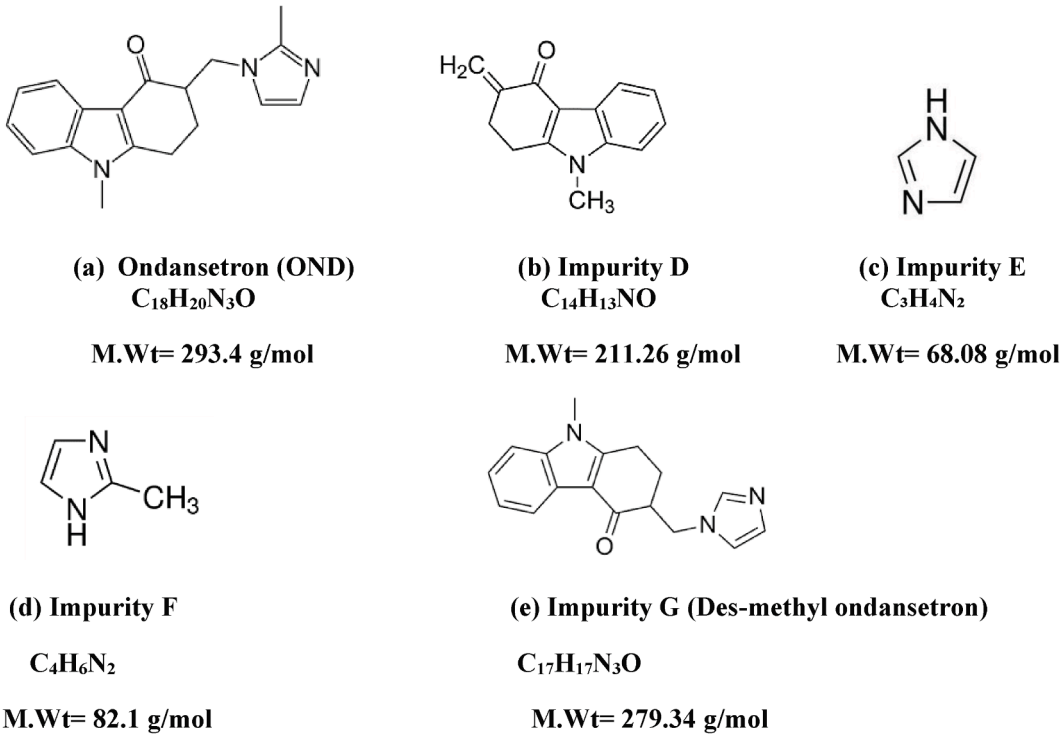


Fig. 1. Chemical structure of Ondansetron and its official impurities.

used and consequently the volume of waste produced [9–11]. Also, the use of buffer in their mobile phases is not preferred as it is a source of microbial growth and decreases the column lifetime [12]. Furthermore, the USP assay [6] separated OND from three impurities only which are A, C, and D.

As per our endeavor to support the sustainability and green analytical chemistry (GAC), an LC-MS/MS method was developed for the analysis of OND along with four selected official potential impurities; namely impurity D, E, F and G (Fig. 1) and HPTLC-densitometry for analysis of OND together with impurity D in pharmaceutical formulations. The LC-MS/MS method has the advantage of separating compounds that have the same retention time and similar absorption spectra in addition it can determine analytes at the nanoscale level. On the other hand, the HPTLC-densitometric method is simple, cost-effective, needs no expensive sophisticated instrument, consumes a small volume of solvents, and is one of the most adaptable and affordable techniques for separation of API from its related compounds or impurities [13–16].

To our knowledge all the published LC/MS methods [17–23] dealt with the determination of OND in biological samples and without addressing the impurities quantitation nor the application to pharmaceutical preparation, Supplementary material Table 1SM. Additionally, it is the first LC/MS/MS method to quantify OND simultaneously with its four official impurities with high sensitivity, low detection limit, high specificity, short run time, and low amount of solvent used when compared to HPLC/UV reported methods. In addition, the software of PreADMET and pkCSM were used for the first time to expect the toxicity profile of OND official impurities.

Moreover, the greenness criteria of the proposed methods were evaluated and compared with reported HPLC/UV methods using four assessment tools; National Environmental Methods Index (NEMI), analytical eco-scale assessment (ESA), Green Analytical Procedure Index (GAPI), Analytical GREEnness Metric Approach (AGREE). The green assessment tools measure the method's environmental impact as the amount of reagents used and their health/ safety hazards, the instrumental energy, the volume of waste produced, and any other occupational hazards. Additionally, the whiteness profiles were evaluated according to the new approach; RGB 12 algorithm. The whiteness takes into account the quality of the developed analytical method in terms of validation criteria, productivity, practicality, and economic effectiveness.

2. Experimental

2.1. Instruments and software

Shimadzu HPLC system is equipped with a Shimadzu SIL20A autosampler, a binary pump (Shimadzu LC20AT), and a triple quadrupole mass spectrometer (API 3200). Analyst 1.6.3 software was used to carry out the data acquisitions.

TLC plates (20 × 20 cm) precoated with silica gel 60 F_{254} (Merck, Germany). A Camag Linomat-5 autosampler (Switzerland) with a microsyringe, UV cabinet, and twin-trough developing chamber. The densitometric measurement was performed by a Camag TLC scanner IV operated with winCATS® software.

Toxicity profiling was carried out using online software; preADMET (https://preadmet.qsarhub.com/toxicity/) and pkcsm (https://biosig.lab.uq.edu.au/pkcsm/). The toxicity profiling involves testing acute algae, acute daphnia, and acute fish toxicity, Ames test for compound mutagenicity testing of various Salmonella typhimurium strains. Additionally, tests for carcinogenicity are conducted using in vitro human ether-a-go-go related gene channel hERG inhibition and carcinogenicity bioassays using mice and rats, respectively. The maximum recommended tolerated dose (MRTD), oral rat acute and chronic toxicity, hepatotoxicity, and skin sensitization were also calculated as toxicity predictors.

2.2. Materials and reagents

Pure standards: OND and its official impurities (D, E, F, and G) were kindly gifted by Sunny Medical Group, Egypt.

Pharmaceutical formulations: Ondalenz® oral films (batch no: 22233) were manufactured by Nerhadou International Company, Egypt.

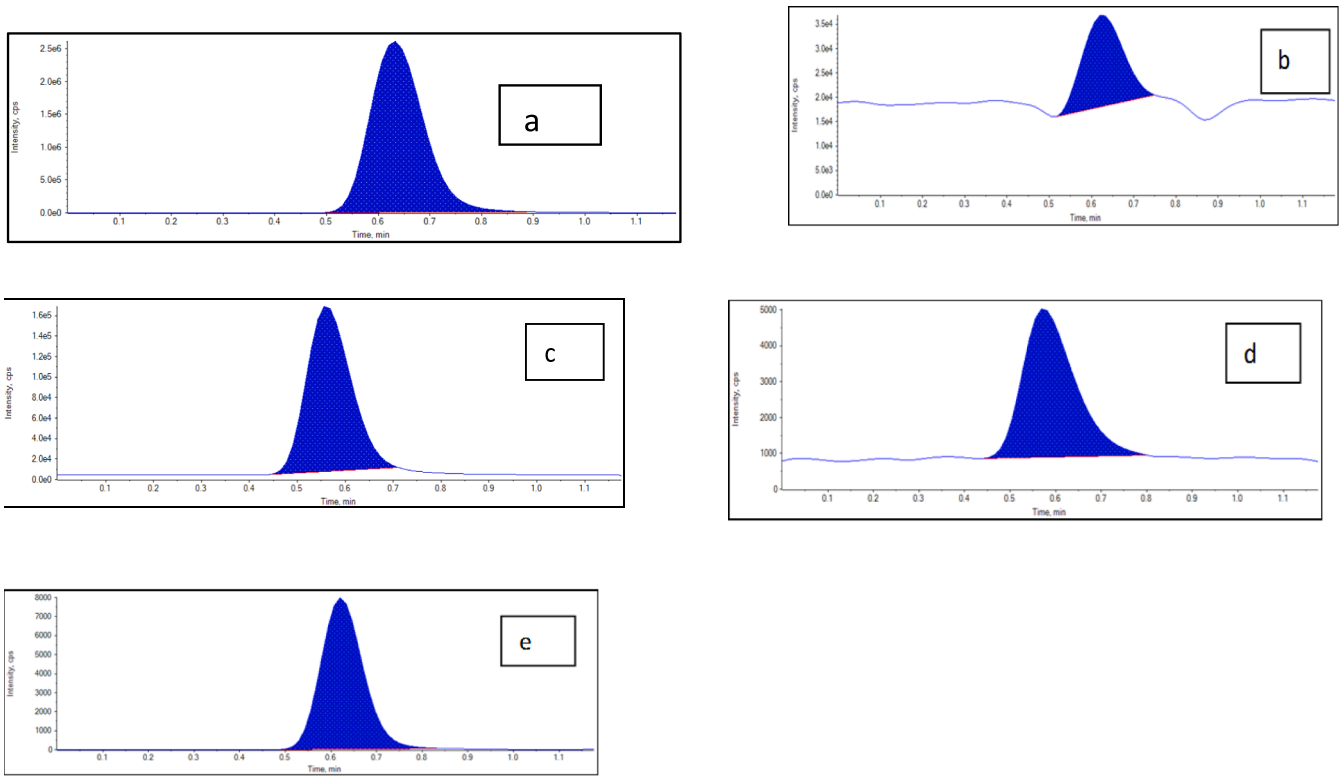


Fig. 2. MRM chromatograms of (a) OND, (b) impurity D, (c) impurity E, (d) impurity F and (e) impurity G.

Each film is labeled to contain 8 mg of OND. Danset® injection (batch no: 201139) was manufactured by ADWIA company, Egypt. Each vial was labeled to contain 8 mg/4mL of OND.

Solvents: methanol of HPLC grade and formic acid (Fischer Scientific UK), ethyl acetate (Sigma, Germany).

2.3. Stock and working standard solutions

Stock solutions of (1 mg/mL) were prepared for each of OND and the impurities using methanol as solvent. The working solutions (0.1 mg/mL) were prepared by further dilution from the stock solutions using methanol.

3. Procedures

3.1. LC-MS/MS and mass spectrometric condition

Inetsil C_{18} column (4.6*50 mm,5μm particle size) was used with isocratic elution of methanol: 0.1 % formic acid in water in a ratio of (70: 30 v/v%) as a mobile phase. The injection volume was 5 μL and the flow rate was set at 1 mL/min with a total run time of 2 min.

Multiple reaction monitoring (MRM) with electrospray ionization (ESI) in positive ions was used to quantify OND and its impurities. The gas temperature was set at 300 °C, de-solvation, and nebulization gas: nitrogen at flow 7 L/min, Nebulizer 15Psi, capillary voltage 4000 V, collision energy 25 V, fragmentary voltage 135 V, and cell accelerator voltage 7 V.

3.2. HPTLC- densitometric conditions

Different volumes of OND and impurities D, E, F, and G were transferred from their working solutions and were applied using the micro-syringe, 1.5 cm apart, onto TLC silica plates (10 x 2 0 cm). The TLC- chamber was allowed to be saturated with the mobile phase for 30 min. The mobile phase consists of ethyl acetate: methanol in a ratio (6:4 v/v) was eluted over the injected plates. The elution time was around 10.0 min. The plates are removed, allowed to dry at room temperature, and scanned at 216 nm.

3.3. System suitability for the HPTLC-densitometry

As per the USP [6], the system suitability parameters were measured to confirm that the technique functions correctly. The values of retardation factor (R_f), resolution (R_s), tailing factor (T), Capacity factor ($\acute{k}$), and selectivity factor (α) were calculated.

3.4. Application to pharmaceutical formulations

Five milliliters from the Danset® injection were transferred into a 10-mL volumetric flask and completed to the volume with methanol. For the Ondalenz®, five oral films were dissolved in methanol and filtered, then a volume equivalent to 10 mg was transferred to a 10-mL volumetric flask; to prepare solutions of 1 mg/mL for each pharmaceutical formulation.

4. Results and discussion

The aim of this research is to establish green, sensitive, and validated LC-MS/MS and HPTLC-densitometric methods for the determination of OND along with its official impurities.

To date, there has been neither a toxicity profiling study on OND impurities nor LC-MS/MS nor HPTLC methods for the determination of OND with its official impurities. Therefore, in the current work, validated LC-MS/MS and HPTLC-densitometric methods were developed for their simultaneous determination in pharmaceutical dosage. In addition, the toxicity profile of OND impurities was evaluated using PreADMET and pkCSM online software.

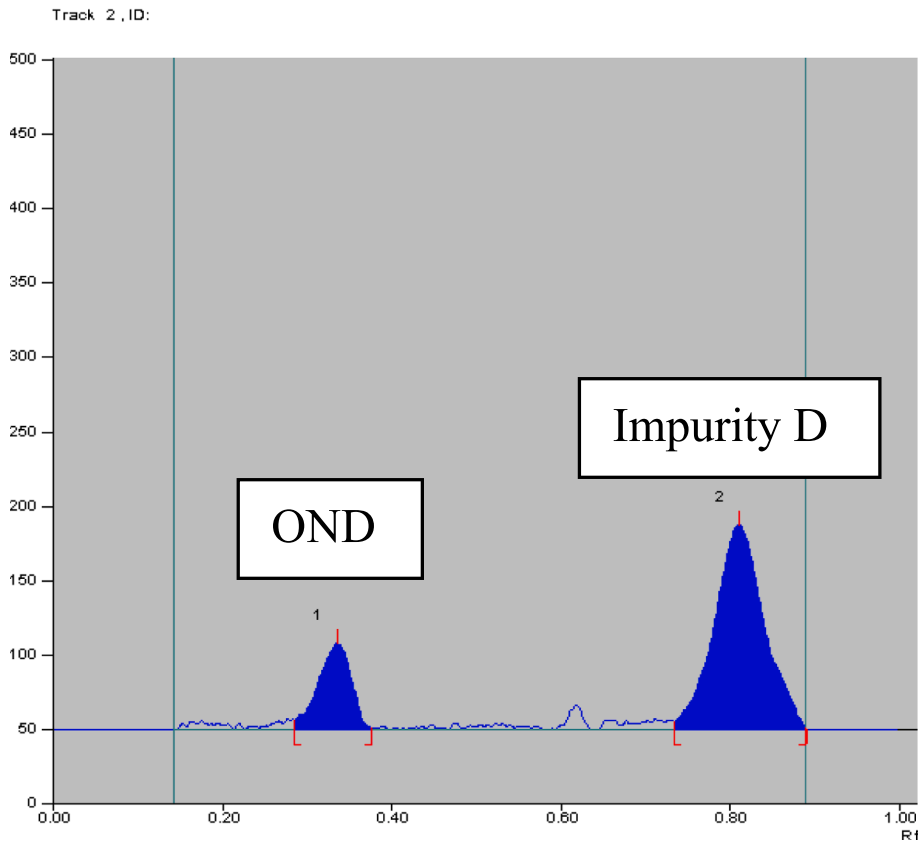


Fig. 3. ^{2}D HPTLC-densitogram of OND (1 μg/spot) ($R_f = 0.33$) and impurity D (0.1 μg/spot) ($R_f = 0.82$), using a developing system of ethyl acetate: methanol (6:4 v/v) and UV detection at 216 nm.

4.1. In silico toxicity profiling of OND impurities

The results for examining the toxicity profiling of OND and its impurities using both preADMET and pKcsm databases are shown in Table 2SM. The preADMET results revealed that impurity, D, showed positive AMES mutagenicity to three salmonella strains and impurity H showed mutagenicity to two strains. Moreover, impurities E and F showed the highest toxicity against algae and daphnia. Only impurity E showed positive carcinogenicity for mice. Also, OND and all impurities have a medium risk for hERG inhibition. Furthermore, the pKcsm results showed low MTRD for all impurities except for OND and impurities F and G, where their values are more than 0.447 log (mg/kg/day). The predicted values for hepatotoxicity are likely to be associated with disrupted normal function of the liver for impurities G and H while the values are not likely to be associated with skin sensitization.

Based on the above results, we can conclude that impurities D, E, and F may represent toxic impurities. Though impurity G, which has no published data up to date, showed similar significant results to OND.

4.2. Development and optimization of LC-MS/MS chromatographic conditions

The developing conditions were chosen to take into consideration the nature of analytes and the method is further optimized to get the best separation, sensitivity, and selectivity.

The composition and pH of the mobile phase had an impact on the separation and ionization. The mobile phase was optimized by comparing water-acetonitrile/methanol and water-containing formic acid-acetonitrile/methanol. The use of Methanol as an organic phase provided better-shaped peaks when compared to acetonitrile it also gave a higher sensitivity with a lower background, in addition, methanol is more eco-friendly than acetonitrile. It was found also that the addition of formic acid to water improved the separation and made the peak sharper, so the Methanol: 0.1 % formic acid mixture was tried in different ratios. Several columns of different dimensions were tried, and the C_{18} Column was found to be the best choice for the separation of impurities E and F. The optimum chromatogram having well-resolved symmetrical peaks was obtained using the C_{18} column (4.6*50 mm, 5 μm) and a mobile phase consisting of methanol: 0.1 % formic acid in a ratio (70:30 v/v) at a flow rate 1 mL/min. Column temperature was set at 40°C for sharp peak shape and constant retention times.

The use of multiple reactions monitoring (MRM) mode ensured the selectivity of the method. The mass spectra were measured in both positive and negative modes to determine the best ionization mode. Positive ion base peak intensities were greater than negative ion base peak intensities. The mass detection was done using MRM mode; m/z 294.1 < 170.0 for OND, m/z 210.4 < 184.4 for impurity D, m/z 68.9 < 44.8 for impurity E, m/z 83.1 < 41.9 for impurity F and m/z 280.35 < 170.3 for impurity G, Supplementary material Fig. 1SM. Fig. 2 showed the MRM chromatograms of the five compounds and the mass spectrometric parameters were listed in Supplementary material Table 3SM.

4.3. Development and optimization of HPTLC-densitometry conditions

Several trials were performed for the separation of OND from its four impurities. The trials focused on altering the mobile phase system using green solvents without decreasing the separation efficiency. Several mobile phase systems were tried, consisting of ethyl acetate, methanol, and *cyclo*-hexane in different ratios with and without the addition of a few drops of ammonia. Impurities E and F did not move from the baseline as they are highly polar. So, we increased the mobile phase polarity by adding toluene to the mixture of (ethyl acetate and methanol) but the two impurities were strongly retained on the silica plate due to their high polarity. Consequently, this method could not detect or

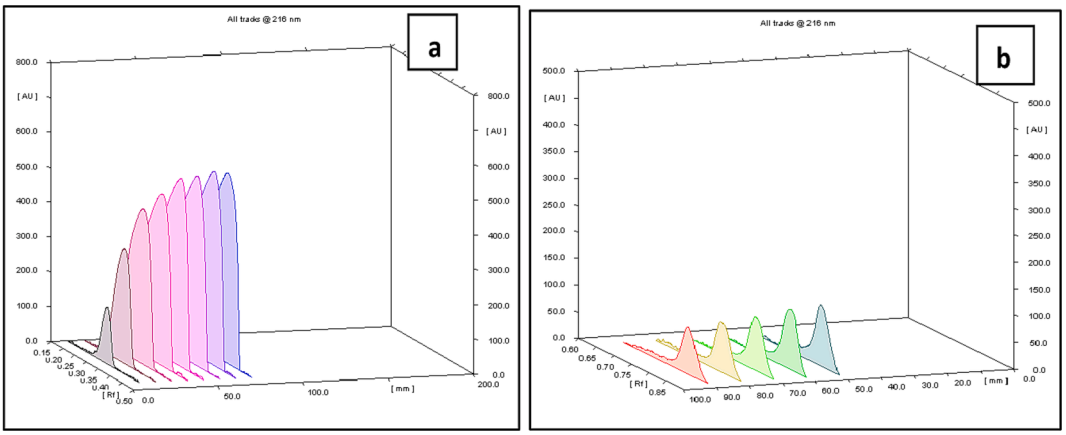


Fig. 4. ^{3}D HPTLC-densitogram for the calibration curve of (a) OND (1–100 μg/spot) and (b) Impurity D (0.1–0.9 μg/spot), using mobile phase consisting of ethyl acetate: methanol (6:4 v/v) and detection at 216 nm.

Table 1
Assay parameters and method validation for the determination of Ondansetron with its official impurities by the proposed chromatographic methods.

Parameter	LC-MS/MS					HPTLC-densitometry	
	OND	Impurity D	Impurity E	Impurity F	Impurity G	OND	Impurity D
Linearity (ng/mL)	2–500	0.2–25	0.2–25	0.2–25	0.05–5	1–100	0.1—0.9
Slope	22.69	11,943	89,663	2932.2	10,781	−15.504[b] 2306.9[c]	−123.57[b] 313.27[c]
SE of the slope	0.3137	413.57	4855.3	72.275	353.18		
Intercept	295.79	1509.1	84,501	3275.6	89.828	9415.2	43.703
SE of intercept	16.938	6634.8	77,711	1156.8	1111.7		
Correlation coefficient (r)	0.9994	0.9982	0.9956	0.9991	0.9984	0.9945	0.9963
Accuracy[a] (mean ± RSD%)	101.87 ± 1.15	100.73 ± 0.73	100.06 ± 0.82	101.37 ± 1.04	100.94 ± 1.23	102.87 ± 1.15	101.73 ± 1.73
LOD (ng/mL)	0.101	0.03	0.02	0.06	0.013	0.05	0.01
LOQ (ng/mL)	0.308	0.07	0.071	0.18	0.041	0.15	0.03
Intra-day Precision[a] (RSD%)	0.74	0.66	0.97	1.10	0.68	1.14	1.66
Inter-day Precision[a] (RSD%)	1.73	1.44	1.32	1.87	1.36	1.83	1.94

Slope *c and b are the coefficients of x^2 and x, respectively for a polynomial regression equation ($y = ax^2 + bx + c$).where, y is the peak area, x is the concentration of the analyte, *a* and *b* are the are the coefficients and *c* is the intercept. [a]Calculated from three determinations.

[a] Calculated from three determinations.

Table 2
Recovery of the impurities in laboratory-prepared mixtures, by the proposed chromatographic methods.

Analyte	Concentration[a] (ng/mL)	Recovery[b] by LC/MS/MS	Concentration[a] (μg/spot)	Recovery[b] by HPTLC
Impurity D	0.2	101.5 ± 1.7	0.1	98.6 ± 2.7
	2.0	97.0 ± 3.2	0.5	94.3 ± 1.5
	5.0	105.2 ± 0.6	0.9	99.5 ± 2.3
Impurity E	0.2	99.5 ± 4.3		
	2.0	102.6 ± 1.6		
	5.0	100.8 ± 1.3		
Impurity F	0.2	101.2 ± 0.5		
	2.0	103.7 ± 2.8		
	5.0	100.4 ± 3.5		
Impurity G	0.05	94.7 ± 2.7		
	0.2	100.3 ± 1.4		
	4.0	99.4 ± 0.5		

[a] Amount of impurities spiked with respect to 200 ng/mL of OND.for LC/MS/MS and 100 μg/spot for HPTLC.

[b] Mean ± RSD% for three determinations.

Table 3
System suitability testing parameters of and HPTLC-densitometric method for determination of Ondansetron with impurity D.

Parameter	OND	Impurity D	Reference values [22]
R_f	0.33 ± 0.02	0.82 ± 0.01	
Tailing factor (T)	0.84	1.16	T = 1 for a symmetric peak
Resolution (R_S)	3.03		>1.5
Capacity factor (k')	2.03	0.219	0–10
Selectivity factor (α)		9.26	> 1

quantify the two impurities. Furthermore, all the trials could not resolve impurity G from OND peak (Rs was less than 1), Supplementary material Fig. 2SM, because their structures are very close; impurity G is C-Desmethyl Ondansetron. Additionally, several wavelengths (308, 310, and 216 nm) were tried to get the highest response. The greenest solvent system consisting of ethyl acetate: methanol (6:4 v/v) can determine OND and Impurity D with retardation factors (R_f) values of 0.33 and 0.82, respectively at 216 nm, as shown in Figs. 3 and 4.

The proposed LC-MS/MS method had several advantages over the reported HPLC-UV methods [9,10], firstly the isocratic elution which is simpler, and lower in cost as there is no need for a particular pump, nor column re-equilibration between consecutive injections when compared to the gradient elution used in the reported methods. Secondly, no buffer is used in the elution solvent contrary to the previously reported methods. Moreover, it is more sensitive with lower detection limits

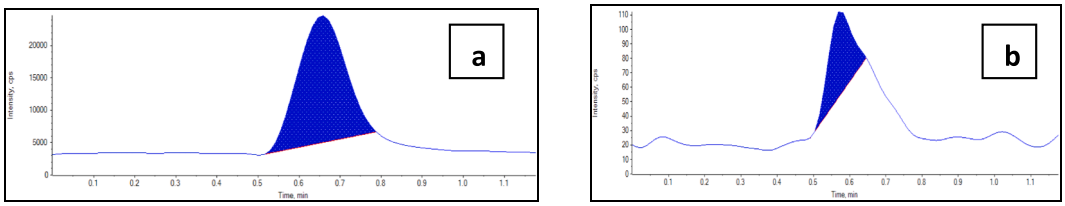


Fig. 5. MRM Chromatogram of (a) impurity D (13 ng/mL) at level 0.05 % and (b) impurity G (0.029 ng/mL) at level 0.0001 %, detected in Danset® injection (25 μg/mL).

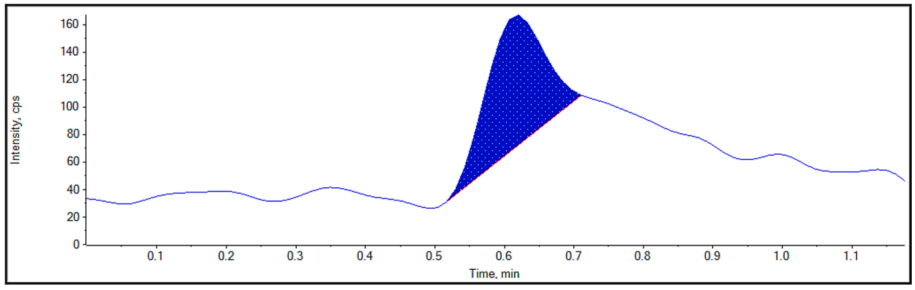


Fig. 6. MRM Chromatogram of impurity G (0.055 ng/mL) at level 0.0002 %, detected in Ondalenz® oral films (25 μg/mL).

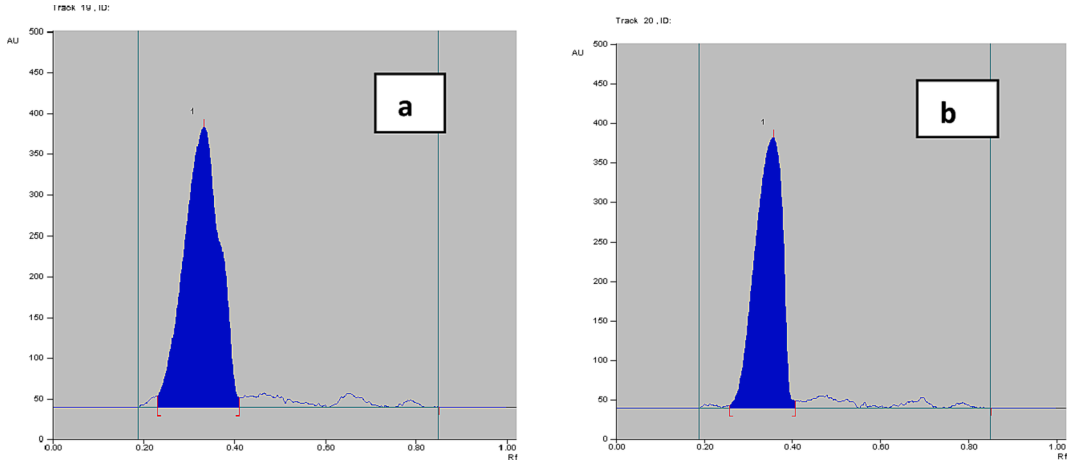


Fig. 7. [2]D HPTLC-densitogram of (a) Danset® injection (5 μg/spot), and (b) Ondalenz® oral films (5 μg/spot), using mobile phase consisting of ethyl acetate: methanol (6:4 v/v) and detection at 216 nm.

Table 4
Determination of Ondansetron in its pharmaceutical formulations and application of standard addition technique using the proposed methods.

Pharmaceutical formulation	LC/MS/MS	HPTLC-densitometry
	Mean recovery %[a] ± SD	
Ondalenz® oral films	100.65 ± 0.47	103.37 ± 1.52
Danset® injection	99.37 ± 0.84	102.65 ± 1.34
Standard addition technique	100.75 ± 0.57	103.83 ± 1.86

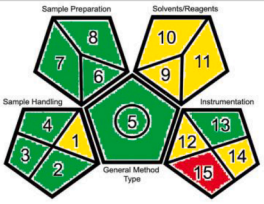
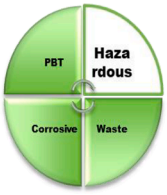
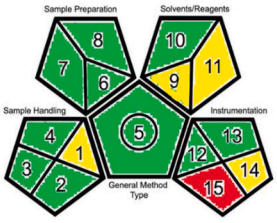
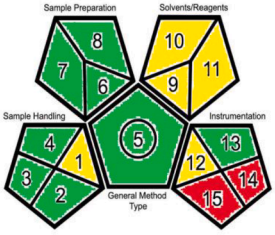
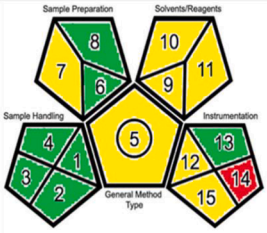
[a] Average of 5 determinations.

which allow the accurate quantification of the impurities that are toxic/mutagenic even if found in very low concentrations (less than 0.1 %). Finally, the shorter run time is due to the ability of the mass detector to determine the assayed analytes with similar spectrum and retention time as it depends on the *m/z* ratio of the compounds, allowing lower solvent consumption and consequently decreasing the waste amount. The HPTLC-densitometric method is considered a greener candidate for analysis as it has lower energy consumption, lower volume of used solvents, and consequently lower waste generation [14,24]. In addition, multiple samples could be analyzed at the same time in a single run, such as 20 samples could be spotted on a plate of dimensions (10 x 20 cm) [25].

4.4. Validation and system suitability parameters of the developed methods

The developed method was validated according to ICH guidelines [26], the parameters verified were linearity, range, accuracy, precision, LOD, LOQ, and specificity. Wide linearity ranges were obtained for OND (of 2–500 ng/mL), (0.2–25 ng/mL) impurities D, E, and F, and (0.05–5 ng/mL) for impurity G regarding LC-MS/MS method. The calibration curves of OND were in the range of (1–100 μg/spot) and impurity D (0.1–0.9 μg/spot) for HPTLC-densitometry. The average peak area is plotted against the corresponding concentration for each compound. A good correlation coefficient between the peak area and concentration of analytes was obtained. The accuracy was assessed by calculating the recovery values which were found within the accepted range. The intra-day and inter-day precision was assessed by measuring three

Table 5
Comparison of the greenness profile for the proposed methods versus the reported chromatographic methods.

Methods	NEMI	ESA		GAPI	AGREE
Proposed LC-MS/MS		Water Methanol Formic acid LC/MS energy Occupational hazard Waste Total penalty points Eco-scale score	0 12 3 2 0 3 ∑ 20 80		
Proposed HPTLC-densitometry		Methanol Ethyl acetate TLC energy Occupational hazard Waste Total penalty points Eco-scale score	12 8 0 0 3 ∑ 23 77		
Reported method I [6]		Methanol NaH_2PO_4 H_3PO_4 LC energy Occupational hazard Waste Total penalty points Eco-scale score	18 0 4 1 0 5 ∑ 28 72		
Reported method II [5]		NaOH ACN NaH_2PO_4 LC energy Occupational hazard Waste Total penalty points Eco-scale score	4 12 0 1 0 5 ∑ 22 78		

concentrations in triplicate within the same day and on three successive days and the RSD % values were found to be less than 2 %.

The LOD and LOQ were calculated using the Signal-to-Noise ratios by injecting standard solutions of known concentrations for each compound for the LC-MS/MS while the HPTLC-densitometry calculation was based on the slope of the calibration curve and standard deviation of the intercept; LOD = 3.3 * (SD of intercept)/Slope, LOQ = 10 * (SD of intercept)/Slope. LC-MS/MS has lower values of LOD and LOQ than HPTLC-densitometry; which makes LC-MS/MS the method of choice because of its sensitivity. The validation parameters for LC-MS/MS and HPTLC are shown in Table 1.

The specificity of the proposed methods was assessed by spiking three levels of each impurity to pure OND reaching 0.1 % level of OND concentration, the recoveries and RSD% were listed in Table 2.

System suitability parameters for the HPTLC-densitometry were found to be conforming to the specified limits, as listed in Table 3. The resolution (R_s) between OND and impurity D was more than 1.5, so the method could be used for their simultaneous analysis. Contrarily, the HPTLC method could not be used for the determination of OND and impurity G, as their peaks are not well resolved as shown in Supplementary material Fig. 2SM.

4.5. Application to pharmaceutical formulations

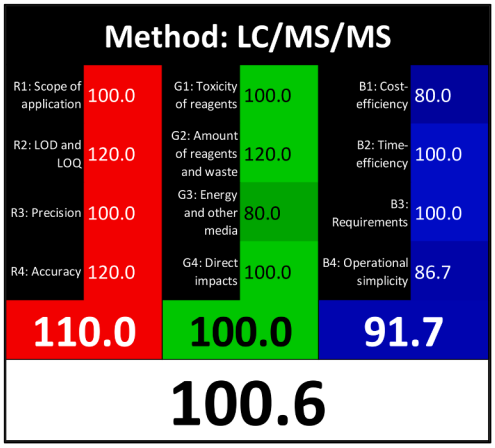
The proposed LC-MS/MS can determine the four impurities in a very minute amount (less than 0.1 %). Impurities E and F were not found in both pharmaceutical formulations (Ondalenz® oral films and Danset® injection). Impurity D was found in Danset® formulation at level 0.05 % but not found in Ondalenz® oral films. Impurity G was present in Ondalenz® oral films at 0.0002 % and in Danset® injection at 0.0001 %, as shown in Figs. 5 and 6.

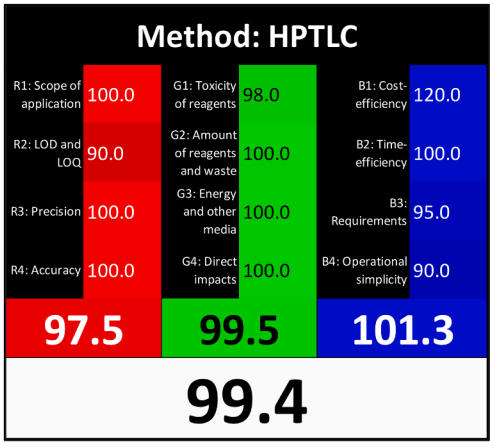
The mentioned levels of impurities were accepted as per ICH guidelines in both formulations. The HPTLC-densitometry did not detect the mentioned minute concentration of impurity D whose cementation was less than 0.1 % as proved by the LC-MS/MS method, Fig. 7, but it succeeded in OND quantification in both formulations. The validity of both methods was confirmed by the standard addition technique. The recoveries and SD values of OND are shown in Table 4.

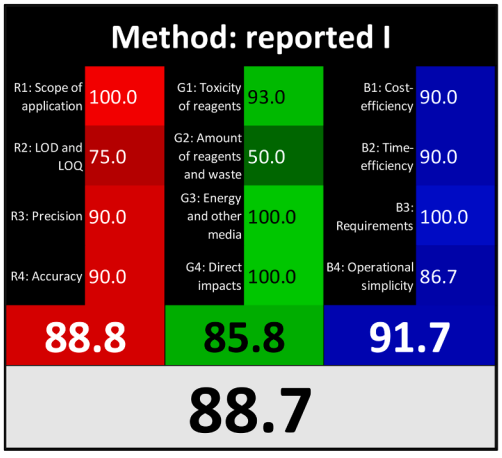
4.6. Green assessment

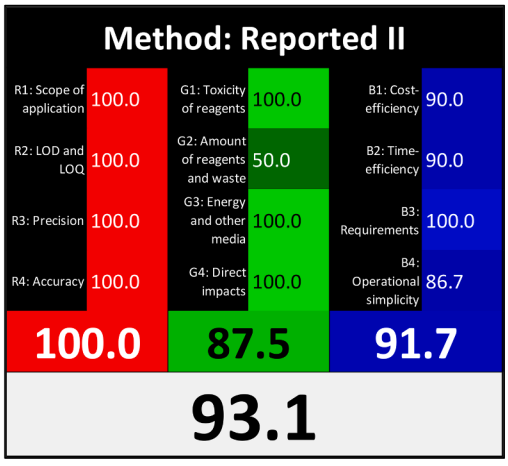
The green character of the established LC-MS/MS and HPTLC-densitometric methods were assessed and also compared with two reported HPLC/UV methods [9,10] by the National Environmental Methods Index (NEMI), Analytical eco-scale assessment (ESA), Green analytical procedure index (GAPI) and Analytical GREEnness Metric

Table 6
Comparison of the Whiteness (%) for the proposed methods versus the reported chromatographic methods according to RGB 12 algorithm.









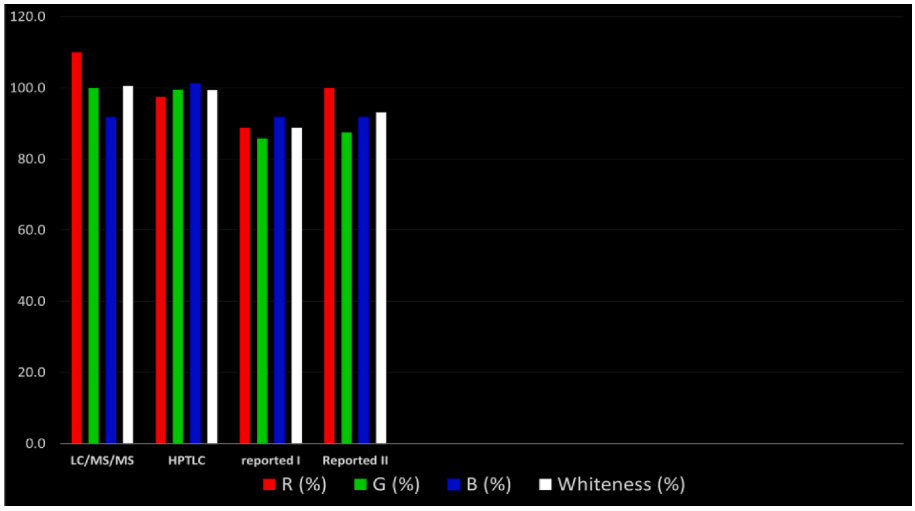


Fig. 8. RGB 12 algorithm for the proposed and reported chromatographic methods.

Approach (AGREE).

The NEMI pictogram of the four methods has the same profile, as presented in Table 5; the hazardous quadrant is blank owing to the use of methanol in the two developed methods and reported method I [10] and acetonitrile in reported method II [9]. The other three quadrants are shaded green as the solvents used are not persistent, bio-accumulative, nor toxic, the pH used in not corrosive ranged from (2–12) and finally, the waste volume did not exceed 50 g/sample. However, this tool is qualitative and cannot differentiate between the green characters of the four methods.

ESA was applied as a semi-quantitative tool to evaluate and compare the greenness to discriminate between the developed and reported

methods. It is based on the calculation of the penalty points and substrate them from 100, the detailed calculations are shown in Supplementary material Table 4SM. The penalty points depended on the hazards of the solvents, the amount of waste produced, instrumental energy, and any other occupational hazards, as shown in Table 5. The two developed methods and reported method II [9] are excellent green methods as their score is higher than 75, reported method I [10] is considered acceptable green with a score of 72.

GAPI was used to evaluate the overall analytical protocols not only the analytical method where all related factors, such as sample extraction, preparation, transport, chemicals, and analytical instruments, are considered. The green color pictograms predominate for the two proposed methods. Even though there are differences in sections 10 and 12; the health hazards of the solvents and the instrumental energy for the proposed LC-MS/MS are higher than that of HPTLC. The reported method II [9] has section 5 shaded yellow as it was considered an indirect method; it requires an extraction procedure before analysis Table 5. The 15 points of comparison according to GAPI tool between the proposed and reported methods were listed in Supplementary material Table 5SM.

The AGREE tool is an automated software that allows an easy, flexible, and straightforward assessment. It is based on the 12 principles of GAC. The overall score is shown in the middle of the circular pictogram, as the score is close to 1 and dark green the procedure is more green [27]. The two developed methods showed higher scores of 0.77 for LC/MS/M and 0.8 for the HPTLC-densitometry than the reported methods which had scores of 0.71 and 0.74. The colored pictograms are presented in Table 5.

In conclusion, using more than one assessment tool is highly recommended when comparing different analytical methods to obtain synergistic results [28–30]. The stated HPLC/UV procedures were shown to be less environmentally friendly than our two newly developed methods as the LC-MS/MS has a shorter run time (2 min) versus more than 18 min and the proposed HPTLC method consumes less volume of solvents and consequently produced less amount of waste and consume less instrumental energy.

4.7. White assessment using RGB 12 algorithm

The newly developed concept of White Analytical Chemistry (WAC) is employed to evaluate the quality of the analytical method in terms of validation efficiency, simplicity, cost, time efficiency, and skills for handling instruments. Besides, it also evaluates the greenness of the method [31–33]. The developed analytical method should be green and equitable (cost-effective and applicable) at the same time. The RGB 12 algorithm is one of the new approaches to assess the whiteness of the method. It is an online freely available Excel spreadsheet and it consists of three principles attributed to three colors: Red - analytical performance, Green- GAC principles, and Blue- sustainability/practical effectiveness the whiteness % is the total of the 3 principles scores [34–36].

Considering the red principle; the proposed LC-MS/MS has the highest score (1 1 0) for analytical performance as it is the most sensitive and accurate method with the lowest LOD and LOQ values, Supplementary material Table 6SM. Regarding the green principle; the two proposed methods had the highest score (100 and 99.5), as was confirmed by the previously mentioned greenness' assessment tools. For the blue model, the proposed HPTLC-densitometry method has the highest score (101.3) as it requires less costly solvents of analytical grades, less time for concomitant analysis, and consequently consumes lower volume of solvents, minimal skills for the handling of instruments and the instrument are less expensive. Hence, it is considered more economical, faster, affordable, and simpler than the other methods. A comparison summary of the reported and proposed chromatographic methods showing that the two developed methods are whiter than the published methods with WAC-based scores of 100.6 and 99.4, respectively, presented in Table 6 and Fig. 8.

5. Conclusion

As a result, the widely used antiemetic drug for cancer patients and pregnant women, OND can be quickly detected and measured in the presence of its toxic/mutagenic impurities in injection and oral film dosage form. This is made possible by sensitive, economical, green, and highly selective chromatographic methods. LC-MS/MS and HPTLC-densitometry were developed and validated according to ICH guidelines. LC-MS/MS succeeded in determining OND without interference from the four impurities (D, E, F, and G) while the HPTLC-densitometric method can determine OND in the presence of impurity D. Five green and white evaluation tools—NEMI, ESA, GAPI, AGREE, and RGB12—were utilized to rate the developed methods. In addition, two online software were applied to evaluate the toxicity profile of the impurities. Based on the research's findings, the suggested methods are better than the reported HPLC/UV methods regarding their greenness, and analytical performance, as well as from an economic and practical standpoint. This impressively supports the capability of the suggested methods to be used for routine quality control.

CRediT authorship contribution statement

Christine M. El-Maraghy: Conceptualization, Supervision, Validation, Writing – original draft, Writing – review & editing. **Mai S. Nour:** Software, Writing – original draft, Writing – review & editing. **Heba T. ELbalkiny:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.110104.

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