ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

A Simple, Sensitive, and Greener HPLC-DAD Method for the Simultaneous Analysis of Two Novel Orexin Receptor Antagonists

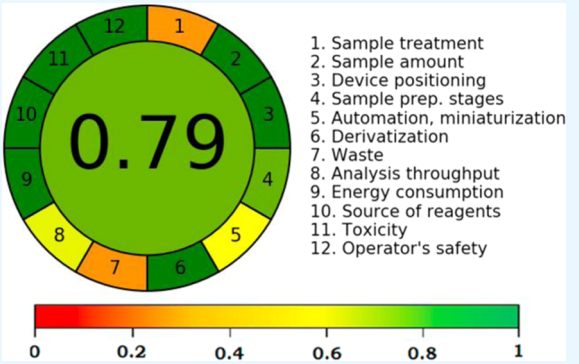
Muzaffar Iqbal,* Nazrul Haq, Mohd Abul Kalam, Faisal Imam, and Faiyaz Shakeel*

Cite This: *ACS Omega* 2024, 9, 23101−23110

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: The orexin receptor antagonist (ORA) is one of the new psychopharmacological agents used in the treatment of insomnia. There are currently no documented greener high-performance liquid chromatography-diode array detector (HPLC-DAD) methods for the analysis of ORA antagonists, lemborexant (LMB) and suvorexant (SUV) simultaneously. Therefore, in this study, a simple, sensitive, and greener HPLC-DAD method has been developed for the simultaneous quantitative analysis of LMB and SUV in bulk and laboratory-prepared mixture. The developed method was validated for numerous validation parameters and evaluated for greenness. The C_{18} Waters Spherisorb CN (4.6 × 250 mm^2; 5 μm) column was used for the chromatographic separation. The mobile phase composition was ethanol: 10 mM KH_2PO_4 buffer in a ratio of (60:40 v/v). The DAD detection was performed at 253 nm using a Waters DAD detector. The greenness was evaluated using the analytical Eco-Scale (AES), ChlorTox, and analytical GREEnness (AGREE) techniques. The calibration curves showed excellent linearity for LMB and SUV between the concentration range of 125−5000 ng/mL and 250−10,000 ng/mL, respectively. In addition, the proposed HPLC-DAD method was accurate, precise, robust, highly sensitive, and greener. AES, ChlorTox, and AGREE scales were predicted by the HPLC-DAD method to be 91, 1.14 g, and 0.79, respectively, showing an excellent greenness profile. The greener HPLC-DAD method was successfully used to analyze both medicines quantitatively in bulk and laboratory-prepared synthetic mixtures. The findings of this study indicated that the proposed HPLC-DAD method may be consistently applied to evaluate LMB and SUV in bulk and dosage forms.



1. INTRODUCTION

Insomnia, the most common sleep-wake disorder, impacts 30 to 50% of the adult global population.[1] Sedatives and hypnotics are the medications that are most frequently recommended to treat insomnia.[2] However, their use is now limited due to their well-known negative consequences, such as dizziness, sleepiness, blurred vision, impaired depth perception, memory loss, depression, etc.[2,3] Recently, it has been suggested that the orexin-1 (OX1) and orexin-2 (OX2) receptors may be a new target therapy of insomnia.[4] For the treatment of adult insomnia, lemborexant (LMB) is the second dual OX receptor antagonist that has been approved.[5] It displays rapid attachment and dissociation from OX1 and OX2 receptors in contrast to earlier dual OX receptor antagonists, enabling rapid and uninterrupted sleep during the night without the possibility of side effects or fatigue the following morning.[6] Suvorexant (SUV) is a potent dual OX1 and OX2 receptor antagonist that blocks the OX neurons of the arousal system, which promote alertness, causing a rapid onset of sleep.[7,8] SUV is also used to treat insomnia.[8,9] The molecular structures of the LMB and SUV are presented in Figure 1. Because of their sedative and hypnotic properties, both drugs are significant medicines from a forensic standpoint.[10,11] Due to their abuse potential, both drugs are classified as a Schedule IV controlled substance.[12,13] The studied drugs LMB and SUV are relatively new compared to other sedatives/hypnotics. Because both medicines are important from a forensic point of view, the adulteration of each other and their illegal use are possible in commercial products. Due to the forensic significance of LMB and SUV, it is quite likely that they will be used illegally; therefore, a sensitive and accurate analytical technique is required to identify them.

A review of the literature found no established analytical techniques for the simultaneous analysis of LMB and SUV in biological samples or dosage forms. There have been reports of certain analytical techniques for the individual analysis of biological materials and commercial formulations containing LMB or SUV. However, the simultaneous analysis of LMB and

Received: April 25, 2024
Accepted: May 9, 2024
Published: May 16, 2024

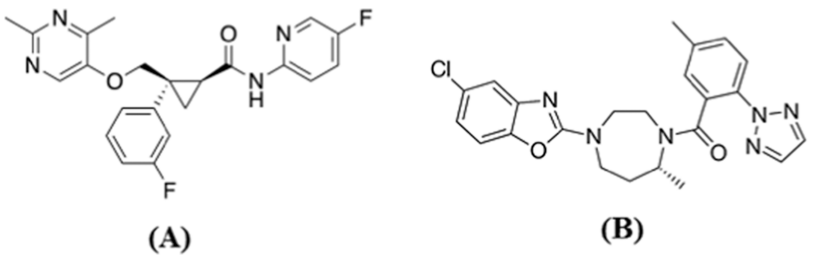


Figure 1. Molecular structures of (A) lemborexant (LMB) and (B) suvorexant (SUV).

SUV has not yet been reported because LMB and SUV are not commercially approved in combined dosage forms. Some high-performance liquid chromatographic (HPLC) approaches have been documented in the literature for the analysis of LMB alone in its dosage forms.[14,15] The simultaneous analysis of LMB and its three metabolites (M4, M9, and M10) in human plasma samples and phosphate-buffered saline has been done using the LC-mass spectrometry (MS)/MS (LC-MS/MS) approach.[16] The literature has documented the use of an ultraperformance LC-MS/MS (UPLC-MS/MS) technology for the simultaneous analysis of LMB and an internal standard, losartan in human plasma samples.[17] Furthermore, the analysis of SUV alone in pharmaceutical products has been described using an HPLC method.[18] There have been reports of the use of certain bioanalytical HPLC methods for the analysis of SUV alone in human and rabbit plasma samples.[19,20] For the analysis of SUV alone in blood and urine samples, reports of the bioanalytical approaches LC-MS/MS[21] and LC-quadrupole/time-of-flight−MS (LC-Q/TOF-MS)[22,23] have also been made. A bioanalytical method utilizing LC-MS/MS has also been documented for the simultaneous analysis of SUV and 16 distinct benzodiazepines in the whole blood specimen.[24] The analysis of SUV alone in plasma samples has been done using certain UPLC-MS/MS bioanalytical techniques.[25,26] SUV, lorcaserin, and brivaracetam have all been reported to be simultaneously analyzed in human plasma samples using a UPLC-MS/MS bioanalytical technique.[27] SUV analysis alone in human urine samples has been done using a few additional bioanalytical techniques,[28] such as gas chromatography-MS (GC−MS)[29] and high-performance thin-layer chromatography (HPTLC).[30]

Utilizing ecologically suitable alternative solvents is one of the 12 tenets of "green analytical chemistry (GAC)" and is intended to lessen the detrimental impacts of hazardous and poisonous eluents on the environment.[31] The usage of greener solvents has increased significantly during the past few decades, according to a literature search.[32−37] Many analytical techniques for assessing the greenness profiles of pharmaceutical analytical methods are described in the literature.[38−46] The "National Environmental Method Index (NEMI),[38] the Environmental Assessment Tool (EAT),[39] the Analytical Method Volume Intensity (AMVI),[40] the Analytical Eco-Scale (AES),[41] the Green Analytical Procedure Index (GAPI),[42] the Analytical Method GREEnness Score (AMGS),[43] Red, Green, and Blue (RGB),[44] ChlorTox,[45] and the Analytical GREEnness (AGREE)[46]" are some examples of these approaches. To examine the greenness of the current methodology, the current study used three different tools: AES,[41] ChlorTox,[45] and AGREE.[46]

As far as we are aware, there have not been any cases of the simultaneous analysis of LMB and SUV in their combination dosage forms and biological samples. Therefore, the goal of the current approach is to create and validate a reversed-phase HPLC-diode array detector (DAD) method that is simple, sensitive, and greener for the simultaneous analysis of LMB and SUV in laboratory-prepared synthetic mixtures. Because both medicines are important from a forensic point of view, their illegal use is possible. The present method will help in the identification of adulteration of each other and hence forensic analysis of both medicines compared to the methods reported for these medicines individually. The proposed method for the simultaneous determination of LMB and SUV was validated by "The International Council for Harmonization (ICH)-Q2-R2" criteria.[47]

2. RESULTS AND DISCUSSION

2.1. Development of the HPLC-DAD Method. A summary of the chromatographic responses that were recorded and the combinations of different greener mobile phases is displayed in Table 1.

Figure 2 displays the representative chromatograms of the blank and standards LMB and SUV. The chromatogram of the blank sample did not display the SUV and LMB peaks (Figure 2A). Because ethanol−water (50:50 v/v), ethanol−water

Table 1. Optimization of Greener Mobile Phase and Recorded Chromatographic Parameters for Standards Lemborexant (LMB) and Suvorexant (SUV) (Mean ± SD, *n* = 3)[a]

greener mobile phase	A_s	N	R_t
	LMB		
ethanol/water (50:50 v/v)	2.74 ± 0.64	2832 ± 2.14	5.71 ± 0.34
ethanol/water (60:40 v/v)	2.56 ± 0.58	3341 ± 2.28	5.62 ± 0.32
ethanol/KH_2PO_4 (50:50 v/v)	1.41 ± 0.12	4314 ± 3.12	4.98 ± 0.08
ethanol/KH_2PO_4 (60:40 v/v)	1.06 ± 0.05	5466 ± 3.87	4.87 ± 0.03
ethanol/ethyl acetate (50:50 v/v)	2.81 ± 0.77	2218 ± 1.88	6.24 ± 0.36
ethanol/ethyl acetate (60:40 v/v)	2.78 ± 0.75	2462 ± 1.94	6.12 ± 0.35
	SUV		
ethanol/water (50:50 v/v)	2.78 ± 0.66	3012 ± 2.27	6.98 ± 0.39
ethanol/water (60:40 v/v)	2.60 ± 0.60	3415 ± 2.38	6.87 ± 0.38
ethanol/KH_2PO_4 (50:50 v/v)	1.43 ± 0.14	4712 ± 3.22	6.72 ± 0.12
ethanol/KH_2PO_4 (60:40 v/v)	1.09 ± 0.06	5871 ± 4.07	6.59 ± 0.04
ethanol/ethyl acetate (50:50 v/v)	2.84 ± 0.81	2378 ± 1.95	7.14 ± 0.44
ethanol/ethyl acetate (60:40 v/v)	2.80 ± 0.77	2554 ± 2.02	7.05 ± 0.42

[a] A_s: peak tailing factor; N: theoretical plates number; R_t: retention time; KH_2PO_4: potassium dihydrogen phosphate.

https://doi.org/10.1021/acsomega.4c03976
ACS Omega 2024, 9, 23101−23110

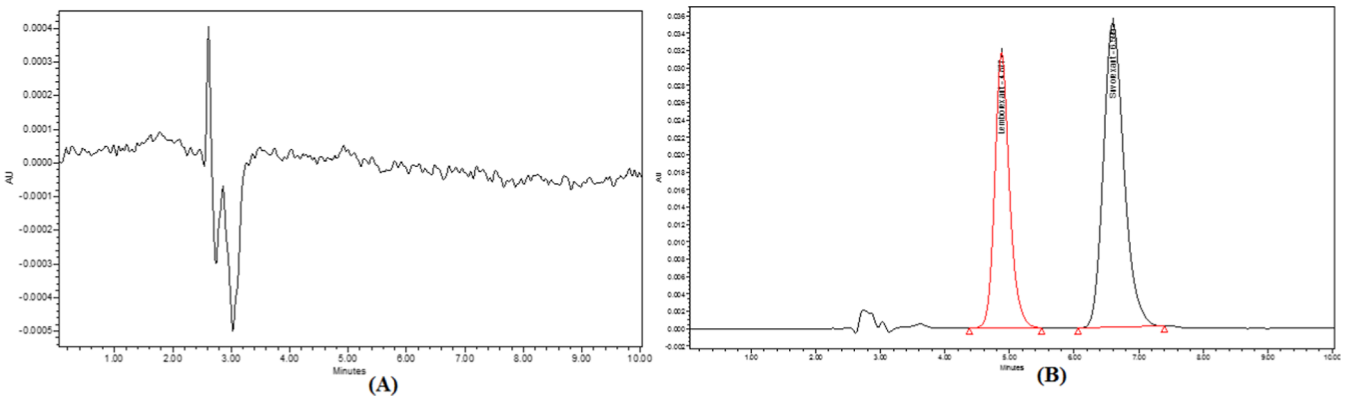


Figure 2. Representative chromatograms of (A) blank and (B) standards LMB (R_t = 4.87 min) and SUV (R_t = 6.59 min) obtained using ethanol/KH_2PO_4 buffer (60:40 v/v) yields a greener mobile phase.

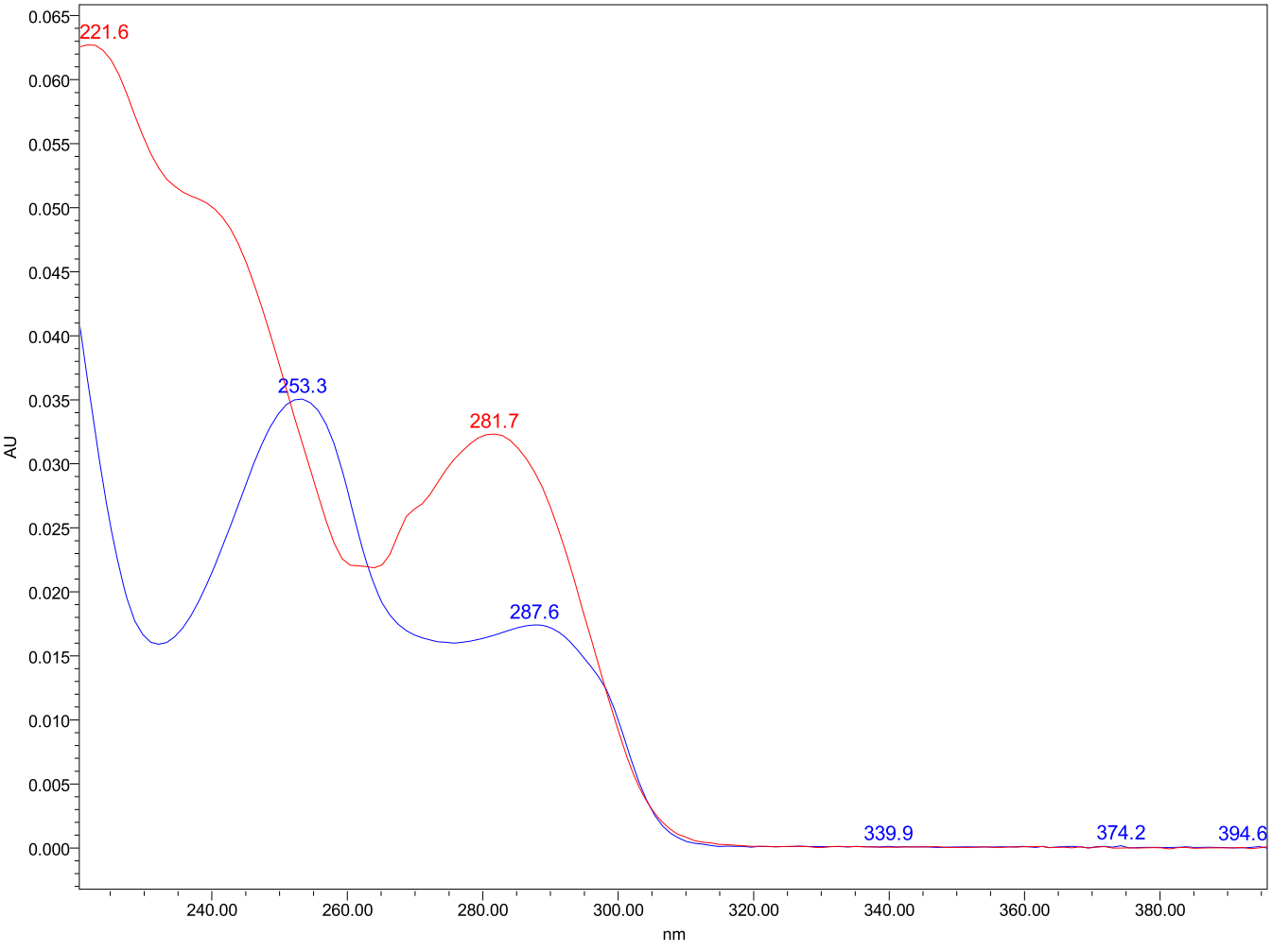


Figure 3. Diode array detector (DAD) spectra for SUV (blue color, λ_{max} = 253.3 nm) and LMB (red color, λ_{max} = 281.7 nm).

(60:40 v/v), ethanol-ethyl acetate (50:50 v/v), and ethanol-ethyl acetate (60:40 v/v) were used during the method development process, the chromatographic parameters of LMB and SUV were inadequate. LMB showed a low number of theoretical plate (N) values (N = 2218−3341) and larger tailing factor (A_s) values (A_s = 2.56−2.78). SUV also showed low N values (N = 2378−3415) and larger A_s values (A_s = 2.60−2.84). Nevertheless, using ethanol-potassium dihydrogen phosphate (KH_2PO_4) buffer in 50:50 and 60:40 v/v ratios as greener mobile phases produced better outcomes with reliable retention time (R_t), A_s, and N values. The ethanol-KH_2PO_4 buffer combination (60:40 v/v), on the other hand, showed well-separated and intact chromatographic peaks of LMB and SUV with a good A_s value and higher N values, and it also produced a consistent R_t (Figure 2B). The final greener mobile phase for determining LMB and SUV simultaneously with an adequate A_s (1.06 for LMB and 1.09 for SUV) and N (5466 for LMB and 5871 for SUV), adequate analysis time (R_t = 4.87

https://doi.org/10.1021/acsomega.4c03976
ACS Omega 2024, 9, 23101−23110

min for LMB and 6.59 min for SUV), and an acceptable analysis time (10 min) was therefore decided to be a binary mixture of ethanol-KH_2PO_4 buffer (60:40 v/v). The DAD wavelengths for the simultaneous estimation of LMB and SUV were screened at the 200−400 nm regions. The individual DAD wavelengths were 281.7 and 253.3 nm, for LMB and SUV, respectively (Figure 3). However, the highest response was obtained at 251 nm for the simultaneous analysis of LMB and SUV. Thus, the full simultaneous determination of the LMB and SUV occurred at 251 nm using the DAD mode.

2.2. Validation of the HPLC-DAD Method. Using the ICH-Q2-R2 standards,[47] a range of parameters were generated for the simultaneous assessment of SUV and LMB. For the simultaneous analysis of LMB and SUV, there are no analytical methods available in the literature. Therefore, the validation parameters of the present study were not compared with literature methods. The results of the greener HPLC-DAD method's linearity assessment of the LMB and SUV calibration curves are shown in Table 2. The LMB and SUV calibration

Table 2. Linearity Measurement Data for the Simultaneous Analysis of LMB and SUV Using the Greener HPLC-DAD Method (Mean ± SD; n = 3)[a]

parameters	LMB	SUV
linear range (ng/mL)	125−5000	250−10,000
regression equation	y = 99,945x + 4882.1	y = 78,096x − 3024.2
R^2	0.9968	1.0000
R	0.9983	1.0000
SE of slope	84.37	76.44
SE of intercept	13.37	9.43
95% CI of slope	99,581.93−100,308.10	77,767.04−78,424.96
95% CI of intercept	4824.56−4939.63	2983.58−3064.82
LOD ± SD (ng/mL)	0.76 ± 0.02	0.68 ± 0.02
LOQ ± SD (ng/mL)	2.29 ± 0.06	2.05 ± 0.06

[a]R^2: coefficient of determination; R: correlation coefficient; x: LMB or SUV concentration; y: LMB or SUV peak area; SE: standard error; CI: confidence interval; LOD: limit of detection; LOQ: limit of quantitation.

curves were linear in the 125−5000 and 250−10,000 ng/mL ranges, respectively. It was projected that the determination coefficient (R^2) for LMB and SUV would be 0.9968 and 1.0000, respectively. It was discovered that the correlation coefficients (R) for SUV and LMB were 1.0000 and 0.9983, respectively. For LMB and SUV, the R^2 and R values were significant ($p < 0.05$). These findings showed a strong relationship between the LMB and SUV concentrations and the observed responses. These results showed that the proposed HPLC-DAD approach was linear enough to determine SUV and LMB simultaneously.

Using R_t, A_s, capacity factor (k), and N, the system compatibility parameters for the developed HPLC-DAD approach were ascertained. The results of the system suitability parameters along with their reference values are included in Table 3. The values of R_t, A_s, k, and N for LMB obtained using the current approach were 4.87 min, 1.06, 2.41, and 5466, in that order. For SUV, the derived values of R_t, A_s, k, and N were 6.59 min, 1.09, 2.46, and 5871, in that order. The obtained values of R_t, A_s, k, and N were within the range of reference/acceptable values.[47] Therefore, the values for measuring LMB and SUV simultaneously were reliable and good.

Table 3. System Suitability Parameters of LMB and SUV for the Greener HPLC-DAD Method (Mean ± SD; n = 3)[a]

parameter	recorded value	reference value	refs
	LMB		
R_t (minutes)	4.87 ± 0.03	>1	47
A_s	1.06 ± 0.05	0.80−1.15	47
k	2.41 ± 0.07	>2	47
N	5466 ± 3.87	>2000	47
	SUV		
R_t (minutes)	6.59 ± 0.04	>1	47
A_s	1.09 ± 0.06	0.80−1.15	47
k	2.46 ± 0.09	>2	47
N	5871 ± 4.07	>2000	47

[a]R_t: retention time, A_s: peak tailing factor, k: capacity factor, N: number of theoretical plates.

The percentage recovery for the simultaneous detection of SUV and LMB was used to assess the accuracy of the developed HPLC-DAD method. Table 4 displays the accuracy

Table 4. Accuracy Data of LMB and SUV for the Proposed HPLC-DAD Method (Mean ± SD; n = 3)[a]

conc. (ng/mL)	conc. found (ng/mL) ± SD	recovery (%)	CV (%)	reference value	refs
		LMB			
750	755.41 ± 7.35	100.72	0.97		
1000	1018.12 ± 9.34	101.81	0.91	100 ± 2	47
1250	1240.24 ± 10.45	99.21	0.84		
		SUV			
1500	1520.21 ± 16.32	101.34	1.07		
2000	1986.21 ± 20.02	99.31	1.00	100 ± 2	47
2500	2478.32 ± 24.62	99.13	0.99		

[a]CV: coefficient of variance.

evaluation findings for the HPLC-DAD method that was developed. Using the greener HPLC-DAD approach, the percentage recoveries of LMB and SUV at three different quality control (QC) levels were found to be, respectively, 99.21−101.81 and 99.13−101.34%. The obtained % recoveries of LMB and SUV were within the acceptable range.[47] These findings showed that the greener HPLC-DAD approach could reliably measure the simultaneous analysis of the LMB and SUV.

For the simultaneous analysis of LMB and SUV, the intraday (repeatability) and interday (reproducibility or intermediate) precision of the greener HPLC-DAD method was assessed; the results are presented as the percent of coefficient of variance (%CV). The accuracy and precision concentrations were selected in such a way that the low QC (LQC), middle QC (MQC), and high QC (HQC) levels could be covered. Therefore, the selected concentrations for the determination of accuracy and precision were the same. The precision findings for the LMB and SUV simultaneous analysis utilizing the current methods are shown in Table 5. For the intraday fluctuation, the percentage CVs of SUV and LMB were found to be 0.87−0.91 and 0.75−0.83%, respectively. For LMB and SUV, the interday variation percentage CVs were found to be 0.81−0.93 and 0.89−0.95%, respectively. The obtained % CVs of LMB and SUV were within the acceptable range.[47] All of these results proved how precise the greener HPLC-DAD method for the concurrent determination of LMB and SUV is.

https://doi.org/10.1021/acsomega.4c03976
ACS Omega 2024, 9, 23101−23110

Table 5. Assessment of Intra/Interday Precision of LMB and SUV for the Greener HPLC-DAD Method (Mean ± SD; n = 3)

	intraday precision			interday precision				
conc. (ng/mL)	conc. (ng/mL) ± SD	standard error	CV (%)	conc. (ng/mL) ± SD	standard error	CV (%)	reference value	refs
			LMB					
750	741.25 ± 6.21	3.58	0.83	757.41 ± 7.10	4.09	0.93		
1000	1014.61 ± 8.12	4.68	0.80	986.42 ± 8.41	4.85	0.85	<2	47
1250	1237.45 ± 9.31	5.37	0.75	1261.32 ± 10.22	5.90	0.81		
			SUV					
1500	1491.31 ± 13.65	7.88	0.91	1517.23 ± 14.56	8.40	0.95		
2000	1982.35 ± 17.45	10.07	0.88	2020.31 ± 18.43	10.64	0.91	<2	47
2500	2530.12 ± 22.12	12.77	0.87	2482.45 ± 22.31	12.88	0.89		

The robustness analysis results at the (MQC levels of SUV and LMB are shown in Table 6. Upon modification of the composition of the greener mobile phase to evaluate robustness, the percentage CVs for SUV and LMB were determined to be 0.98−1.15 and 0.86−1.08%, respectively. The R_t for LMB and SUV were derived to be 4.86−4.88 and 6.57−6.61 min, respectively. The % CVs for LMB and SUV were determined to be 0.87−1.09 and 0.94−1.18%, respectively, in the event that the flow rate was altered during a robustness analysis. The R_t for LMB and SUV were found to be 4.72−4.99 and 6.41−6.70 min, respectively. The % CVs were found to be 0.88−1.10 and 0.95−1.08%, respectively, in the event that DAD wavelength was altered during a robustness analysis. The R_t for LMB and SUV were found to be 4.85−4.89 and 6.56−6.60 min, respectively. The robustness of the current approach for measuring LMB and SUV concurrently is indicated by low CVs and a negligible R_t value change.

Table 6. Robustness Measurement Results of LMB and SUV for the Greener HPLC-DAD Method (Mean ± SD; n = 3)

parameters	conc. found (ng/mL) ± SD	CV (%)	R_t ± SD
	LMB		
mobile phase (ethanol/KH_2PO_4, v/v)			
(62:38)	984.56 ± 8.51	0.86	4.86 ± 0.03
(58:42)	1016.91 ± 11.02	1.08	4.88 ± 0.04
flow rate (mL/min)			
(1.10)	1019.12 ± 11.18	1.09	4.72 ± 0.02
(0.90)	978.28 ± 8.52	0.87	4.99 ± 0.04
DAD wavelength (nm)			
255	994.18 ± 8.76	0.88	4.85 ± 0.02
251	1022.41 ± 11.28	1.10	4.89 ± 0.05
	SUV		
mobile phase (ethanol/KH_2PO_4, v/v)			
(62:38)	1986.12 ± 19.65	0.98	6.57 ± 0.04
(58:42)	2023.61 ± 21.34	1.15	6.61 ± 0.05
flow rate (mL/min)			
(1.10)	2028.12 ± 23.97	1.18	6.41 ± 0.03
(0.90)	1979.63 ± 18.68	0.94	6.70 ± 0.04
DAD wavelength (nm)			
255	1984.85 ± 18.91	0.95	6.56 ± 0.04
251	2032.22 ± 22.08	1.08	6.60 ± 0.06

The sensitivity of the greener HPLC-DAD method for the concurrent analysis of LMB and SUV was evaluated as the limit of detection (LOD) and limit of quantification (LOQ). Table 2 shows the LOD and LOQ values that were determined for LMB and SUV using the greener HPLC-DAD approach. Using the greener HPLC-DAD method, the LOD and LOQ for LMB were derived to be 0.76 ± 0.02 and 2.29 ± 0.06 ng/mL, respectively. Using the greener HPLC-DAD method, the LOD and LOQ for SUV were derived to be 0.68 ± 0.02 and 2.05 ± 0.06 ng/mL, respectively. These results showed that the greener HPLC-DAD approach was quite sensitive when it came to measuring SUV and LMB simultaneously. These results showed that the greener HPLC-DAD approach was quite sensitive when it came to measuring SUV and LMB simultaneously.

2.3. Application of Greener HPLC-DAD Method in the Simultaneous Determination of LMB and SUV in Laboratory-Prepared Synthetic Mixtures. Analytical techniques for the simultaneous determination of SUV and LMB have not been reported in the literature. As a result, the current study's pharmaceutical assay results were not contrasted with those of previous research. For the simultaneous analysis of LMB and SUV in a laboratory-prepared synthetic mixture, the greener HPLC-DAD method was used as an alternative to traditional HPLC methods. By contrasting the HPLC chromatograms of LMB and SUV to those of standards LMB and SUV utilizing the greener HPLC-DAD method, we recognized the chromatograms of LMB and SUV from laboratory-prepared synthetic mixtures were recognized. The representative chromatograms of LMB and SUV in the laboratory-prepared synthetic mixtures are presented in Figure 4. These chromatograms were found to be identical to those of the standards for LMB and SUV (Figure 2B). In addition, there were no additional peaks of the excipients in the formulation chromatogram, indicating that the studied drugs LMB and SUV did not show interactions with the formulation excipients. The % assay of LMB and SUV in laboratory-prepared synthetic mixtures was computed using the greener HPLC-DAD method, and the results were 98.12 ± 1.16 and 101.31 ± 1.23%, respectively. These results proved that the greener HPLC-DAD method is suitable for measuring LMB and SUV in laboratory-prepared synthetic mixtures simultaneously.

2.4. Greenness Assessment. Developed analytical methods can be assessed for their greenness using a variety of approaches such as NEMI,[38] EAT,[39] AMVI,[40] AES,[41] GAPI,[42] AMGS,[43] RGB,[44] ChlorTox,[45] and AGREE.[46] The greenness of the greener HPLC-DAD method was evaluated in the current work by using three distinct approaches: AES,[41] ChlorTox,[45] and AGREE.[46]

https://doi.org/10.1021/acsomega.4c03976
ACS Omega 2024, 9, 23101−23110

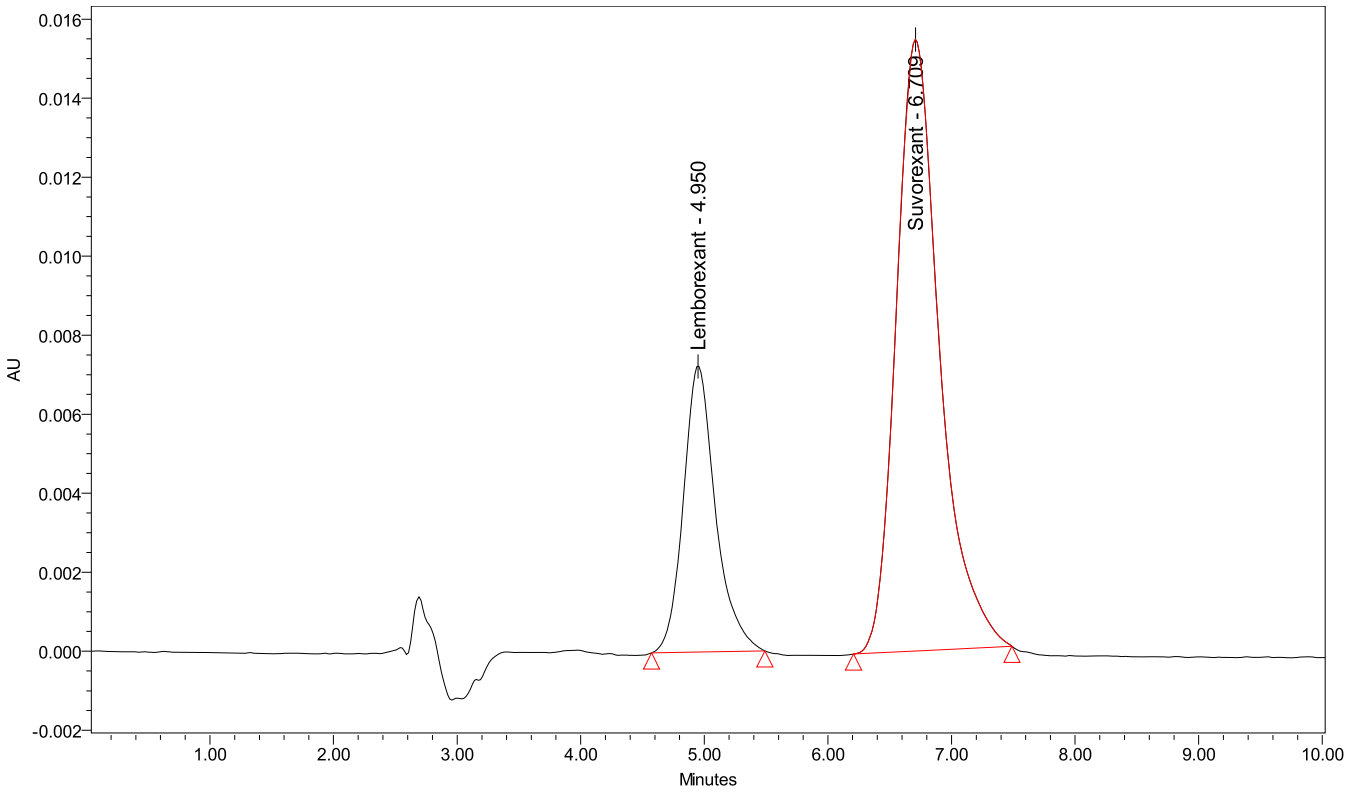


Figure 4. Representative chromatogram of LMB and SUV in laboratory-prepared synthetic mixtures.

For the simultaneous analysis of LMB and SUV, no documented analytical methods are available. Therefore, the greenness parameters of the present study were not compared with literature methods. AES is a useful semiquantitative method that takes waste, instruments, and all analytical stages into account. Table 7 shows the results of the AES scales with penalty points. Less than 50 on the scale denoted insufficient greenness, less than 75, but greater than 50 on the scale denoted appropriate greenness, and greater than 75 on the AES rating denoted extraordinary greenness.[41] The AES scale for the current approach was found to be 91, which indicates an extraordinary greenness profile.

Table 7. Analytical Ecoscale (AES) and Penalty Point Evaluation for the Greenness of the Proposed HPLC-DAD Method

parameters	penalty points
reagents	
ethanol	4
KH_2PO_4 (10 mM)	0
instruments	
energy consumption	0
occupational hazard	0
waste	5
total penalty points	9
AES score	91

For the greener HPLC-DAD approach, Table 8 shows the total ChlorTox as well as the results of the different solvent ChlorTox scales. The greener HPLC-DAD method's estimated total ChlorTox scale was 1.14 g, indicating that it was both relatively safe and environmentally friendly.[45]

The AGREE technique, which takes into account each of the 12 GAC criteria,[46] is the most commonly used quantitative method for evaluating greenness. Figure 5 displays the overall AGREE scale for the current HPLC-DAD methodology. An AGREE scale greater than 0.75 denoted excellent greenness, a scale less than 0.75 denoted sufficient greenness, and a value less than 0.50 denoted inadequate greenness.[46] The overall AGREE scale was predicted by environmentally friendly HPLC-DAD technology to be 0.79. The AGREE results once again illustrated the superior green characteristics of the present HPLC-DAD methodology. The greener HPLC-DAD approach for the simultaneous detection of LMB and SUV in synthetic mixtures created in the laboratory has an excellent green profile according to the overall results of all greenness measurements.

Table 8. Data for the ChlorTox Scales of the Greener HPLC-DAD Approach in Terms of the Relative Dangers Concerning Chloroform (CH_{sub}/CH_{CHCl_3}) Calculated Using the WHN Model

stage	solvent/reagent	relative hazard (CH_{sub}/CH_{CHCl_3})	m_{sub} (mg)	ChlorTox (g)	total ChlorTox (g)
sample preparation	ethanol	0.26	552	0.14	1.14
HPLC analysis	ethanol	0.26	3866	1.00	

https://doi.org/10.1021/acsomega.4c03976
ACS Omega 2024, 9, 23101−23110

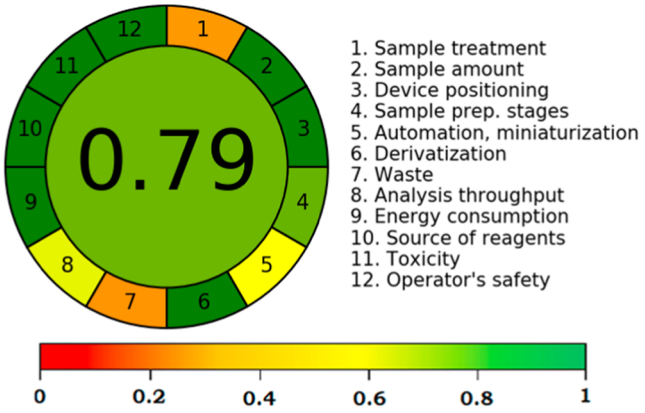


Figure 5. Analytical GREEnness (AGREE) scale for the present HPLC-DAD methodology, driven by an AGREE calculator.

3. CONCLUSIONS

For the simultaneous analysis of LMB and SUV, no documented analytical methods are available. Therefore, this work developed and validated a simple, sensitive, and greener HPLC-DAD method for the simultaneous analysis of LMB and SUV in laboratory-prepared synthetic mixtures. The greener HPLC-DAD method is linear, accurate, precise, robust, incredibly sensitive, and greener when used to determine the LMB and SUV simultaneously. The LMB and SUV contents of synthetic combinations made in the lab were successfully analyzed using the current HPLC-DAD technique. The results of the AES, ChlorTox, and AGREE assessments confirm the outstanding greenness of the existing HPLC-DAD method for detecting LMB and SUV concurrently. All of these results suggested that the proposed HPLC-DAD approach can be routinely employed for the simultaneous measurement of SUV and LMB to determine the illegal use of LMB or SUV and for forensic analysis. Further studies can be performed to analyze LMB and SUV in biological materials such as plasma, blood, and urine in order to evaluate the illegal use of LMB or SUV.

4. MATERIALS AND METHODS

4.1. Materials. The working standards of LMB and SUV were obtained from Beijing Mesochem Technology Co. Ltd. (Beijing, China). The HPLC-grade solvents, such as ethanol and ethyl acetate, were obtained from E-Merck (Darmstadt, Germany). The HPLC-grade water was obtained from the Milli-Q (Milli-Q, Lyon, France) apparatus. The KH_2PO_4 buffer was prepared in the laboratory. The commercial tablets of LMB (each containing 10 mg of LMB) and SUV (each containing 10 mg of SUV) were obtained from (Mumbai, India). AR grade was utilized for all of the other reagents and solvents.

4.2. Instrumentation and Chromatographic Conditions. LMB and SUV were quantified simultaneously at 25 ± 1 °C using the Waters 2690D HPLC system (Waters, Milford, MA), which included a column oven, an inline vacuum degasser, a 717 automated sampler, a Waters 996 DAD detector, and an isocratic pump (1515). The data processing and interpretation tool utilized was the Empower 3 software (Milford, MA). A Spherisorb (diameters: 250 mm × 4.6 mm, particle size: 5 μm) RP C_{18} analytical column was used to analyze LMB and SUV concurrently. The greener mobile phase was a binary combination of ethanol and KH_2PO_4 buffer (10 mM) (60:40% v/v) with orthophosphoric acid added to adjust the pH of the mobile phase to 3.5. The greener mobile phase was pumped at a rate of 1 mL/min. It was found that the DAD wavelength of 253 nm was appropriate for measuring LMB and SUV simultaneously. Each sample was injected with a 10 μL injection volume using a Waters autosampler.

4.3. Calibration Curves and QC Samples for LMB and SUV. To create separate batches of LMB and SUV stock solutions, the necessary quantities of each medication were dissolved in the suitable volume of the binary mixture of ethanol and KH_2PO_4 buffer (60:40 v/v)/greener mobile phase. The final stock solution of each medicine comprised 100 μg/mL of the drug. A variety of LMB concentrations between 125 and 5000 ng/mL and SUV values between 250 and 10,000 ng/mL were obtained through the use of the greener eluent system to dilute different amounts of the stock solutions. The peak area of each concentration of LMB and SUV was measured after 10 μL of each concentration was applied by using the Waters autosampler. By plotting the concentrations of LMB and SUV against the observed peak area in three replications (n = 3), LMB and SUV calibration curves were created. Three separate QC samples were made from scratch in order to assess the various validation parameters.

4.4. Sample Processing for the Simultaneous Analysis of LMB and SUV in Laboratory-Prepared Synthetic Mixtures. The commercial tablets of LMB and SUV are not available in combined dosage forms. Therefore, the synthetic mixture of LMB and SUV was prepared in the laboratory to determine LMB and SUV simultaneously. Twenty commercial tablets of LMB (each containing 10 mg of LMB) and SUV (each containing 10 mg of SUV) were taken, and the average weight was computed separately for each commercial tablet. After being roughly crushed, the commercial tablets LMB and SUV were powdered. The 10 mL of the greener mobile phase was mixed with a fine powder that weighed an equal amount of LMB and SUV tablets. 50 mL of the greener mobile phase was added to 1 mL of this solution to make it suitable for the greener HPLC-DAD technique. To get rid of any insoluble impurities, the produced solution was filtered and subjected to sonication for approximately 10 min. Using the greener HPLC-DAD approach, the acquired samples were used to evaluate LMB and SUV simultaneously in synthetic mixtures of LMB and SUV that were produced in the lab.

4.5. Development of the HPLC-DAD Method. Several combinations of green solvents were investigated as the mobile phases in order to create a simple, sensitive, and greener HPLC-DAD approach for the simultaneous detection of LMB and SUV in synthetic mixtures generated in the lab. Ethanol–water, ethanol-KH_2PO_4 buffer, and ethanol-ethyl acetate were three of the numerous green solvent combinations that were investigated. When selecting the greener solvent system, factors such as the solvent cost, greenness, toxicity, sensitivity of the method, length of analysis, measurement parameters, and compatibility of the solvents were taken into account. Many green solvent combinations were therefore considered to be used as mobile phases. In the end, it was decided that the ideal eluent system for further research would be a 60:40 (v/v) blend of ethanol and KH_2PO_4 buffer.

4.6. Validation of the HPLC-DAD Method. Using the ICH-Q2-R2 validation criteria, the proposed HPLC-DAD technique for the simultaneous assessment of LMB and SUV was validated for a number of parameters.[47] Plotting the LMB and SUV concentrations against the observed peak area yielded their linear ranges. Three replicates had their LMB and SUV

https://doi.org/10.1021/acsomega.4c03976
ACS Omega 2024, 9, 23101−23110

linearity (n = 3) evaluated in the ranges of 125−5000 and 250−10,000 ng/mL, respectively.

R_t, A_s, k, and N were established as the system suitability parameters for the environmentally friendly HPLC-DAD method.[48,49]

Using the spiking/standard addition methodology, the accuracy of the greener HPLC-DAD method for the simultaneous detection of LMB and SUV was assessed as a percentage of recoveries.[47] In order to establish LQC solutions of LMB of 750 ng/mL, MQC levels of 1000 ng/mL, and HQC levels of 1250 ng/mL, the previously measured LMB solution (500 ng/mL) was spiked with additional 50, 100, and 150% LMB solutions. To get LQC solutions of 1500 ng/mL, MQC levels of 2000 ng/mL, and HQC levels of 2500 ng/mL, extra 50, 100, and 150% SUV solutions were spiked into the previously measured SUV solution (1000 ng/mL). The commercial tablets of LMB and SUV in combined dosage forms are not available in the market. Therefore, the real samples were not used for the spiking. To assess the accuracy, the LMB and SUV QC solutions from earlier were reexamined. To determine the % recovery at each LMB and SUV level, three replicates (n = 3) were used.

For the purpose of measuring SUV and LMB simultaneously, the greener HPLC-DAD method was assessed for precision in both the intra-assay (repeatability) and interassay (intermediate or reproducibility). Quantifying newly produced LMB and SUV samples at the aforementioned QC levels on the same day (n = 3) allowed for the examination of intra-assay variance for LMB and SUV. A three-day period (n = 3) of freshly generated solution assessment at the previously indicated QC levels was carried out in order to determine the interassay variance for LMB and SUV utilizing the greener HPLC-DAD method. The % CV was used to express both precisions.

In order to determine the impact of deliberate alterations on the simultaneous detection of LMB and SUV, the robustness of the environmentally friendly HPLC-DAD approach was assessed. To assess robustness, the MQC thresholds of SUV (2000 ng/mL) and LMB (1000 ng/mL) were chosen. By alteration of the DAD wavelength, flow rate, and composition of the greener mobile phase, robustness was assessed. When the original ethanol: KH_2PO_4 buffer (60:40 v/v) greener mobile phase was substituted with ethanol: KH_2PO_4 buffer (62:38 v/v) and ethanol: KH_2PO_4 buffer (58:42 v/v), the changes in chromatographic response were recorded. To assess robustness, the starting flow rate of 1 mL/min was adjusted to 1.10 and 0.90 mL/min. In order to assess the robustness of the results, the chromatographic response was observed at other DAD wavelengths (253 and 251 nm). The variations in the chromatographic response were noted.

The sensitivity of the greener HPLC-DAD method for the simultaneous assessment of LMB and SUV was determined as LOD and LOQ by using a standard deviation approach. Equations 1 and 2 (n = 3) were utilized to calculate LMB and SUV LOD and LOQ[47]

$$\mathrm{LOD} = \frac{3.3 \times \sigma}{S} \tag{1}$$

$$\mathrm{LOQ} = \frac{10 \times \sigma}{S} \tag{2}$$

where S is the slope of the calibration curve for LMB and SUV, and σ is the standard deviation of the intercept.

4.7. Greenness Assessment. The greener HPLC-DAD method to measure LMB and SUV concurrently was evaluated for its greenness profile using three distinct approaches: AES,[41] ChlorTox,[45] and AGREE.[46] AES is a semiquantitative technique that takes into account all of the analytical steps, waste, and tools. For the solvents/reagents that need minimal to no reagent use, little energy, and no waste, an ideal analysis with 100 points is predicted. If any of these requirements are broken, penalty points are given and subtracted from the total of 100[41]. Equation 3[45] is used to determine the ChlorTox scale in accordance with the ChlorTox scale technique.

$$\mathrm{ChlorTox} = \frac{\mathrm{CH_{sub}}}{\mathrm{CH_{CHCl_3}}} \times m_{\mathrm{sub}} \tag{3}$$

where $\mathrm{CH_{sub}}$ represents the chemical risks of the substance of interest, $\mathrm{CH_{CHCl_3}}$ is the chemical hazard of standard chloroform, and m_{sub} is the mass of the substance of interest required for a single analysis. The safety data sheet provided by Sigma-Aldrich (St. Louis, MO) was utilized to help in the computation of the values of $\mathrm{CH_{sub}}$ and $\mathrm{CH_{CHCl_3}}$ using the weighted hazards number (WHN) model.[45] The AGREE-metric approach was used to gauge the AGREE scale for the greener HPLC-DAD method for the simultaneous analysis of LMB and SUV.[46] The AGREE scales for the greener HPLC-DAD method were determined using the AGREE: The Analytical Greenness Calculator (version 0.5, Gdansk University of Technology, Gdansk, Poland, 2020). The values ranged from 0.0 to 1.0 based on 12 different GAC principles.

AUTHOR INFORMATION

Corresponding Authors

Muzaffar Iqbal − *Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*; orcid.org/0000-0001-5821-5600; Email: muziqbal@ksu.edu.sa

Faiyaz Shakeel − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*; orcid.org/0000-0002-6109-0885; Email: fsahmad@ksu.edu.sa

Authors

Nazrul Haq − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Mohd Abul Kalam − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Faisal Imam − *Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c03976

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are thankful to the Researchers Supporting Project number (RSPD2024R734), King Saud University, Riyadh, Saudi Arabia, for supporting this work.

REFERENCES

(1) Chung, K. F.; Yeung, W. F.; Ho, F. Y.; Yung, K. P.; Yu, Y. M.; Kwok, C. W. Cross-cultural and comparative epidemiology of insomnia: The Diagnostic and statistical manual (DSM), International classification of diseases (ICD) and International classification of sleep disorders (ICSD). *Sleep Med.* **2015**, *16*, 477−482.

(2) Asnis, G. M.; Thomas, M.; Henderson, M. A. Pharmacotherapy treatment options for insomnia: A primer for clinicians. *Int. J. Mol. Sci.* **2016**, *17*, No. E50.

(3) Sateia, M. J.; Buysse, D. J.; Krystal, A. D.; Neubauer, D. N.; Heald, J. L. Clinical practice guideline for the pharmacologic treatment of chronic insomnia in adults: An American academy of sleep medicine clinical practice guideline. *J. Clin. Sleep Med.* **2017**, *13*, 307−349.

(4) Kumar, A.; Chanana, P.; Choudhary, S. Emerging role of orexin antagonists in insomnia therapeutics: An update on SORAs and DORAs. *Pharmacol. Rep.* **2016**, *68*, 231−242.

(5) Scott, L. J. Lemborexant: First approval. *Drugs* **2020**, *80*, 425−432.

(6) Beuckmann, C. T.; Suzuki, M.; Ueno, T.; Nagaoka, K.; Arai, T.; Higashiyama, H. In vitro and in silico characterization of lemborexant (E2006), a novel dual orexin receptor antagonist. *J. Pharmacol. Exp. Ther.* **2017**, *362*, 287−295.

(7) Bennett, T.; Bray, D.; Neville, M. W. Suvorexant, a dual orexin receptor antagonist for the management of insomnia. *Pharm. Ther.* **2014**, *39*, 264−266.

(8) Patel, K. V.; Aspesi, A. V.; Evoy, K. E. Suvorexant: a dual orexin receptor antagonist for the treatment of sleep onset and sleep maintenance insomnia. *Ann. Pharmacother.* **2015**, *49*, 477−483.

(9) Ahmed, Z.; Ahmad, A.; Khan, S. A.; Husain, A. Pharmacological, pharmaceutical and safety profile of suvorexant: a dual orexin receptors antagonist for treatment of insomnia. *Int. Educ. Sci. Res. J.* **2015**, *1*, 26−30.

(10) Keks, N. A.; Hope, J. Lemborexant, an orexin receptor antagonist sedative-hypnotic: Is it useful for insomnia in psychiatric disorders? *Australas. Psychiatry* **2022**, *30*, 530−532.

(11) Silva-Bessa, A.; Forbes, S. L.; Ferreira, M. T.; Dinis-Oliveira, R. J. Toxicological analysis of drugs in human mummified bodies and proposed guidelines. *Curr. Drug Res. Rev.* **2023**, *15*, 62−72.

(12) U.S. Drug Enforcement Administration. Schedules of controlled substances: placement of suvorexant into Schedule IV. Final rule. *Fed. Regist.* **2014**, *79*, 51243−51247.

(13) Landry, I.; Hall, N.; Aluri, J.; Filippov, G.; Reyderman, L.; Setnik, B.; Henningfield, J.; Moline, M. Abuse potential of lemborexant, a dual orexin receptor antagonist, compared with zolpidem and suvorexant in recreational sedative users. *J. Clin. Psychopharmacol.* **2022**, *42*, 365−373.

(14) Muralikrishna, M.; Nagavalli, S.; Anjali, P.; Deep, P. B.; Teja, D.; Naik, J. P. K. Method development and validation of lemborexant in bulk and its pharmaceutical dosage form by reverse phase−high performance liquid chromatography (RP-HPLC). *World J. Pharm. Res.* **2020**, *9*, 1372−1380, DOI: 10.20959/wjpr202014-19178.

(15) Kamble, S. N.; Munipalli, V. K.; Talapadatur, H.; Singh, R. M.; Warde, S.; Nayak, S.; Vaidhum, B. Development and validation of novel HPLC method for analytical evaluation of lemborexant in tablet dosage form. *GSC Adv. Res. Rev.* **2022**, *11*, 132−143.

(16) Mano, Y.; Ueno, T.; Hotta, K. Establishment of a simultaneous assay for lemborexant, a novel dual orexin receptor antagonist, and its three metabolites, and its application to a clinical protein binding study. *J. Pharm. Biomed. Anal.* **2020**, *187*, No. 113359.

(17) Iqbal, M.; Alshememry, A.; Imam, F.; Kalam, M. A.; Akhtar, A.; Ali, E. A. UPLC-MS/MS based identification and quantification of a novel dual orexin receptor antagonist in plasma samples by validated SWGTOX guidelines. *Toxics* **2023**, *11*, No. E109.

(18) Siddhartha, S.; Ratna, J. V.; Santosh, T. Development and validation of HPLC method for the determination of suvorexant in pharmaceutical dosage forms. *Int. J. Pharm. Drug Anal.* **2018**, *6*, 425−434.

(19) Breidinger, S. A.; Simpson, R. C.; Mangin, E.; Woolf, E. J. Determination of suvorexant in human plasma using 96-well liquid-liquid extraction and HPLC with tandem mass spectrometric detection. *J. Chromatogr. B* **2015**, *1002*, 254−259.

(20) Siddhartha, S.; Ratna, J. V.; Tata, S. K. Development and validation of high performance liquid chromatographic method for the determination of suvorexant in rabbit plasma by HPLC-UV detection. *J. Emerg. Technol. Innov. Res.* **2019**, *6*, 194−203.

(21) Skillman, B.; Kerrigan, S. Identification of suvorexant in blood using LC-MS-MS: Important considerations for matrix effects and quantitative interferences in targeted assays. *J. Anal. Toxicol.* **2020**, *44*, 245−255.

(22) Sullinger, S.; Bryand, K.; Kerrigan, S. Identification of suvorexant in urine using liquid chromatography-quadrupole/time-of-flight mass spectrometry (LC-Q/TOF-MS). *J. Anal. Toxicol.* **2017**, *41*, 224−229, DOI: 10.1093/jat/bkw132.

(23) Skillman, B.; Kerrigan, S. Quantification of suvorexant in blood using liquid chromatography-quadrupole/time of flight (LC-Q/TOF) mass spectrometry. *J. Chromatogr. B* **2018**, *1091*, 87−95.

(24) Garcia, L.; Tiscione, N. B.; Yeatman, D. T.; Richards-Waugh, L. Novel and Nonroutine benzodiazepines and suvorexant by LC-MS-MS. *J. Anal. Toxicol.* **2021**, *45*, 462−474.

(25) Iqbal, M.; Ezzeldin, E.; Khalil, N. Y.; Al-Rashood, S. T. A.; Al-Rashood, K. A. Simple and highly sensitive UPLC-ESI-MS/MS assay for rapid determination of suvorexant in plasma. *J. Anal. Toxicol.* **2017**, *41*, 114−120, DOI: 10.1093/jat/bkw111.

(26) Xu, R.-A.; Chen, K.-L.; Jiao, Y.; Huo, X.-L.; Zhang, Y. Quick method for the determination of suvorexant in plasma. *Lat. Am. J. Pharm.* **2017**, *36*, 2185−2189.

(27) Iqbal, M.; Khalil, N. Y.; Ezzeldin, E.; Al-Rashood, K. A. Simultaneous detection and quantification of three novel prescription drugs of abuse (suvorexant, lorcaserin and brivaracetam) in human plasma by UPLC-MS-MS. *J. Anal. Toxicol.* **2019**, *43*, 203−211.

(28) Iqbal, M.; Ezzeldin, E.; Khalil, N. Y.; Alam, P.; Al-Rashood, K. A. UPLC-MS/MS determination of suvorexant in urine by a simplified dispersive liquid-liquid micro-extraction followed by ultrasound assisted back extraction from solidified floating organic droplets. *J. Pharm. Biomed. Anal.* **2019**, *164*, 1−8.

(29) Carson, M.; Kerrigan, S. J. Quantification of suvorexant in urine using gas chromatography/mass spectrometry. *J. Chromatogr. B* **2017**, *1040*, 289−294.

(30) Alqarni, M. H.; Iqbal, M.; Foudah, A. I.; Aljarba, T. M.; Bar, F. A.; Alshehri, S.; Shakeel, F.; Alam, P. Quantification of suvorexant in human urine using a validated HPTLC bioanalytical method. *ACS Omega* **2023**, *8*, 39928−39935.

(31) Gałuszka, A.; Migaszewski, Z.; Namiesnik, J. The 12 principles of green analytical chemistry and the significance mnemonic of green analytical practices. *Trends Anal. Chem.* **2013**, *50*, 78−84.

(32) Abdelrahman, M. M.; Abdelwahab, N. S.; Hegazy, M. A.; Fares, M. Y.; El-Sayed, G. M. Determination of the abused intravenously administered madness drops (tropicamide) by liquid chromatography in rat plasma; an application to pharmacokinetic study and greenness profile assessment. *Microchem. J.* **2020**, *159*, No. E105582, DOI: 10.1016/j.microc.2020.105582.

(33) Alam, P.; Ezzeldin, E.; Iqbal, M.; Anwer, M. K.; Mostafa, G. A. E.; Alqarni, M. H.; Foudah, A. I.; Shakeel, F. Ecofriendly densitometric RP-HPTLC method for determination of rivaroxaban in nanoparticle formulations using green solvents. *RSC Adv.* **2020**, *10*, 2133−2140.

(34) Alam, P.; Salem-Bekhit, M. M.; Al-Joufi, F. A.; Alqarni, M. H.; Shakeel, F. Quantitative analysis of cabozantinib in pharmaceutical dosage forms using green RP-HPTLC and green NP-HPTLC methods: A comparative evaluation. *Sustainable Chem. Pharm.* **2021**, *21*, No. E100413.

(35) Foudah, A. I.; Shakeel, F.; Alqarni, M. H.; Alam, P. A rapid and sensitive stability-indicating green RP-HPTLC method for the quantitation of flibanserin compared to green NP-HPTLC method: Validation studies and greenness assessment. *Microchem. J.* **2021**, *164*, No. E105960.

https://doi.org/10.1021/acsomega.4c03976
ACS Omega 2024, 9, 23101−23110

(36) Arabi, M.; Ostovan, A.; Li, J.; Wang, X.; Zhang, Z.; Choo, J.; Chen, L. Molecular imprinting: Green perspectives and strategies. *Adv. Mater.* **2021**, *33*, No. E2100543.
(37) Wang, Y.; Li, J.; Sun, D.; Yang, S.; Liu, H.; Chen, L. Strategies of dispersive liquid-liquid microextraction for coastal zone environmental pollutant determination. *J. Chromatogr. A* **2021**, *1658*, No. E462615.
(38) Keith, L. H.; Brass, H. J.; Sullivan, D. J.; Boiani, J. A.; Alben, K. T. An introduction to the national environmental methods index. *Environ. Sci. Technol.* **2005**, *39*, 173A−176A.
(39) Gaber, Y.; Tornvall, U.; Kumar, M. A.; Ali Amin, M.; Hatti-Kaul, R. HPLC-EAT (Environmental Assessment Tool): a tool for profiling safety, health and environmental impacts of liquid chromatography methods. *Green Chem.* **2011**, *13*, 2021−2025.
(40) Hartman, R.; Helmy, R.; Al-Sayah, M.; Welch, C. J. Analytical method volume intensity (AMVI): a green chemistry metric for HPLC methodology in the pharmaceutical industry. *Green Chem.* **2011**, *13*, 934−939.
(41) Gałuszka, A.; Konieczka, P.; Migaszewski, Z. M.; Namiesnik, J. Analytical eco-scale for assessing the greenness of analytical procedures. *TrAC Trends Anal. Chem.* **2012**, *37*, 61−72.
(42) Płotka-Wasylka, J. A new tool for the evaluation of the analytical procedure: Green analytical procedure index. *Talanta* **2018**, *181*, 204−209.
(43) Hicks, M. B.; Farrell, W.; Aurigemma, C.; Lehmann, L.; Weisel, L.; Nadeau, K.; Lee, H.; Moraff, C.; Wong, M.; Huang, Y.; Ferguson, P. Making the move towards modernized greener separations: introduction of the analytical method GREEnness score (AMGS) calculator. *Green Chem.* **2019**, *21*, 1816−1826.
(44) Nowak, P. M.; Koscielnaik, P. What color is your method? Adaptation of the RGB additive color model to analytical method evaluation. *Anal. Chem.* **2019**, *91*, 10343−10352.
(45) Nowak, P. M.; Wietecha-Posluszny, R.; Plotka-Wasylka, J.; Tobiszewski, M. How to evaluate methods used in chemical laboratories in terms of the total chemical risk? a ChlorTox Scale. *Green Anal. Chem.* **2023**, *5*, No. E100056.
(46) Pena-Pereira, F.; Wojnowski, W.; Tobiszewski, M. AGREE-Analytical GREEnness metric approach and software. *Anal. Chem.* **2020**, *92*, 10076−10082.
(47) International Conference on Harmonization (ICH), Q2 (R2) (2023) Guideline on validation of analytical procedures−text and methodology, Geneva, Switzerland.
(48) Haq, N.; Alanazi, F. K.; Salem-Bekhit, M. M.; Rabea, S.; Alam, P.; Alsarra, I. A.; Shakeel, F. Greenness estimation of chromatographic assay for the determination of anthracycline-based antitumor drug in bacterial ghost matrix of *Salmonella typhimurium*. *Sustainable Chem. Pharm.* **2022**, *26*, No. E100642.
(49) Haq, N.; Alshehri, S.; Alam, P.; Ghoneim, M. M.; Hasan, Z.; Shakeel, F. Green analytical chemistry approach for the determination of emtricitabine in human plasma, formulations, and solubility study samples. *Sustainable Chem. Pharm.* **2022**, *26*, No. E100648.

https://doi.org/10.1021/acsomega.4c03976
ACS Omega 2024, 9, 23101−23110