ORIGINAL ARTICLE

A C G publications

J. Chem. Metrol. 17:1 (2023) 15-24

journal of chemical metrology

Simultaneous estimation of remogliflozin etabonate and vildagliptin in a tablet formulation: UV spectrophotometric and HPLC-PDA method

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(Received January 31 20, 2023; Revised March 04, 2023; Accepted March 05, 2023)

Abstract: The ultraviolet spectrophotometric and reversed-phase high-performance liquid chromatography methods were developed and validated to quantify remogliflozin etabonate and vildagliptin in a fixed-dose tablet formulation. The materials and methods used in this study involved using methanol as the solvent in developing three ultraviolet spectrophotometric techniques, namely simultaneous equation, absorption ratio, and derivative spectroscopy. The wavelength maxima for vildagliptin and remogliflozin etabonate were determined to be 232 nm and 217 nm, respectively. The chromatographic method involved using a Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) and a mobile phase consisting of a mixture of methanol and acetate buffer (pH 5.6) in a 70:30 ratio. The flow rate was set at 1 mL/min, and the detecting wavelength was 210 nm. The results obtained from the optimized approaches met the performance test criteria set by the International Conference on Harmonization guideline Q2 (R1). The methods were then applied to assay marketed tablet formulations, and the results obtained were within acceptable limits. In conclusion, the developed techniques can be used to analyze the fixed-dose tablet formulation regularly.

Keywords: FDC; remogliflozin etabonate, HPLC-PDA, T2DM, vildagliptin.

1. Introduction

The formulation containing single-dose combinations of two or more active drugs is known as fixed-dose combinations (FDCs). They are generally preferred due to their improved adherence by lowering the number of required pills and simplifying the dosing regimen with a reduced dose frequency [1-3]. Recently, CDSCO approved a film-coated tablet containing remogliflozin etabonate (100 mg) and vildagliptin (50 mg) as an FDC for treating T2DM. Remogliflozin etabonate (RE) is a novel, patent-protected, and globally researched sodium-dependent glucose cotransporter inhibitor, whereas vildagliptin (VLD) is a dipeptidyl peptidase-4 inhibitor [4-8].

Remogliflozin etabonate (RE) is a prodrug of remogliflozin with a chemical formula of $C_{26}H_{38}N_2O_9$. Meanwhile, vildagliptin (VLD), a white crystalline powder with the chemical formula of

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The article was published by ACG Publications
http://www.acgpubs.org/journal/journal-of-chemical-metrology January-June 2023 EISSN:1307-6183
DOI:http://doi.org/10.25135/jcm.82.2301.2693
Available online: March 10, 2023

$C_{17}H_{25}N_3O_2$, has been used to treat type 2 diabetes mellitus. Figure S1 illustrates the chemical structure of RE (1a) and VLD (1b) in supporting information.

Various analytical approaches, such as spectroscopy, chromatography, and electroanalysis, have been published for the individual quantitation of RE and VLD, as well as their combination, according to a literature review [9-15]. However, only two analytical methods have been reported for the combined dosage form. One method utilized gradient mode RP-HPLC and was applied to a synthetic mixture prepared by the standard addition method using RE (100 mg), VLD (100 mg), and Talc (q.s. 1000 mg). However, this method did not represent the approved dose by the CDSCO for the mentioned FDC [16]. In another method, HPTLC was developed and applied to marketed formulations to carry out the assay [17]. Therefore, there is a need to develop HPLC-PDA and UV spectroscopic methods to quantify the recently approved FDCs of RE and VLD. Despite the reported UV and HPLC methods for analyzing said compounds, their comparative effectiveness and accuracy in determining the purity and concentration of compounds remain an area of interest. In this study, the author aims to comprehensively compare the UV and HPLC methods for determining the purity and concentration of a range of FDC of RE and VLD to understand their relative strengths and weaknesses clearly.

As a result, this study aims to create HPLC-PDA and UV spectroscopic methods to assess both drugs in a combined dosage form simultaneously. PDA can concurrently detect the entire UV-Vis spectrum, making it convenient to find the most suitable wavelength for analysis without repeated analyses. All performance parameters were examined according to ICH rules, and the developed approach was validated.

2. Experimental

2.1. Materials

Pure RE (99.3 %) and VLD (99.7 %) were obtained as free samples from Torrent Pharmaceuticals in Ahmedabad, India, and Aalidhra Pharmachem Pvt. Ltd., Vadodara, India. Apollo Pharmacy provided the Remo V tablet formulation (Ahmedabad, India). Methanol (HPLC-grade) and OPA (ortho-phosphoric acid) were bought from Merck India. Throughout the investigation, Milli-Q water (prepared in-house) was used.

2.2. Instrumentation and Optimized Chromatographic Conditions

In this research, Shimadzu LC-20AT HPLC manual system equipped with binary gradient pumps, a column thermostat, and a PDA detector was used to develop the HPLC method. The stationary phase used was Phenomenex Luna C18 (250 x 4.6 mm, 5 µm), while the mobile phase consisted of 70:30 % v/v methanol: acetate buffer (adjusted to pH 5.6 using OPA). The flow rate was set to 1 mL/min, and the injection volume was 20 µL. Detection was performed at 210 nm using a PDA detector, and the elution mode was isocratic mode. The temperature was maintained at 25º C, and Shimadzu LC-solution software (Version 1.25) was used for data analysis. These instrument settings were critical in ensuring the accuracy and precision of the developed HPLC method for the intended analysis.

2.3. Working Solutions Preparation

To a separate volumetric flask (100 mL), a quantity of 20 mg RE and 10 mg VLD was weighed and transferred. Methanol (60 mL) was added to each flask and sonicated for 10 minutes to dissolve the drug. Finally, the volumes were adjusted to 100 mL using methanol. One milliliter of each resulting mixture was withdrawn and transferred to a new volumetric flask (100 mL) to obtain the working standard. Methanol was added to each flask to make up the volume. The resulting working solutions were 20 µg/mL RE and 10 µg/mL VLD.

2.4. Determination Wavelength Maxima

UV-Vis double beam spectrophotometer was calibrated first to zero absorbance. The working solutions were scanned between 200 and 800 nm for the wavelength maxima. Figure S2 represents the overlain spectra of both drugs (see supporting information).

2.5. Development of Analytical Methods

2.5.1. Simultaneous Equation Method

Using methanol as the diluent, standard solutions (n=3) of RE (10 to 60 µg/mL) and VLD (5 to 30 µg/mL) were prepared. The sample solution equivalent to the target concentration (as per label claim) of RE (20 µg/mL) and VLD (10 µg/mL) was prepared using methanol. Both the absorbances of the solutions were measured at 232 nm and 217 nm. Table 1 shows the data about the simultaneous equation method.

Table 1. Absorptivity values using UV spectroscopic method

Concentration (µg/mL)	Simultaneous equation method		Q-absorbance ratio method		Derivative spectroscopic method	
	Absorbance at λ_1 (232 nm)	Absorbance at λ_2 (217 nm)	Absorbance at λ_1 (217 nm)	Absorbance at λ_2 (243 nm)	Absorbance at λ_1 (232 nm)	Absorbance at λ_2 (217 nm)
			RE			
10	0.323	0.390	0.390	0.038	0.006	0.00
20	0.628	0.752	0.752	0.077	0.005	0.0017
30	0.941	1.024	1.057	0.113	0.023	0.001
40	1.269	1.284	1.284	0.148	0.034	0.013
50	1.529	1.557	1.557	0.179	0.043	0.017
	ax1= 30.5	ax2= 28.7	ax1=28.7	ax2=3.5		
			VLD			
5	0.065	0.295	0.295	0.015	0.00	0.009
10	0.084	0.371	0.371	0.027	0.002	0.022
15	0.102	0.464	0.464	0.04	0.002	0.038
20	0.123	0.558	0.558	0.053	0.006	0.056
25	0.143	0.639	0.639	0.067	0.007	0.072
	a_{Y1}=3.9	a_{Y2}=17.5	a_{Y1}=17.5	a_{Y2}=2.6		

The equations (1) and (2) were used to obtain the drug concentration of the sample solution:

$$C_x = \frac{A2ay1 - A1ay2}{ax2ay1 - ax1ay2} \quad \text{..... (1)} \qquad C_y = \frac{A1ax2 - A2ax1}{ax2ay1 - ax1ay2} \quad \text{..... (2)}$$

The concentration in µg/mL of RE (Cx) and VLD (Cy) was computed using A1 (sample solution absorbance at 232 nm), A2 (sample solution absorbance at 217 nm), ax1 (molar absorptivity of RE at 232 nm), ax2 (molar absorptivity of RE at 217 nm), ay1 (molar absorptivity of VLD at 232 nm) and ay2 (molar absorptivity of VLD at 217 nm).

2.5.2. Q-Absorbance Method

As per the method, the quantitative assay of the analyte in a mixture is determined by measuring the absorbance at two wavelengths, one being the λmax of VLD (217 nm) and the other being a wavelength of equal absorptivity of two drugs (243 nm). The following equations determine the individual concentration of each drug:

$$C_x = \frac{\left(\frac{A2}{A1}\right) - \left(\frac{aY2}{aY1}\right)}{\left(\frac{aX2}{aX1}\right) - \left(\frac{aY2}{aY1}\right)} x \frac{A1}{ax1} \quad \text{..... (3)} \qquad Cy = \frac{\left(\frac{A2}{A1}\right) - \left(\frac{aX2}{aX1}\right)}{\left(\frac{aY2}{aY1}\right) - \left(\frac{aX2}{aX1}\right)} x \frac{A1}{ay1} \quad \text{..... (4)}$$

The concentration in µg/mL of RE (Cx) and VLD (Cy) was computed using A1 (sample solution absorbance at 217 nm), A2 (sample solution absorbance at 243 nm), ax1 (molar absorptivity of RE at 217 nm), ax2 (molar absorptivity of RE at 243 nm), ay1 (molar absorptivity of VLD at 217 nm) and ay2 (molar absorptivity of VLD at 243 nm).

2.5.3. Derivative Spectroscopic Method

The first-derivative UV spectra for RE and VLD solutions were acquired in a 5 mm cell across 200-400 nm using methanol as blank. Each spectrum was captured on film. RE and VLD zero-crossing points were noted. By changing the concentrations of both medications, characteristic wavelengths (ZCPs) for RE and VLD were confirmed. The cells were replenished with a fresh solution after each replicate measurement. The following equations were used to determine the individual concentration of each drug:

$$y1=0.0009x1-0.0037$$
$$y2=0.0032x2-0.0086$$

where,

y1 and *y2* are the absorbances in derivative mode of the spectra.
x1 and *x2* are the concentrations of RE and VLD, respectively.

2.5.4. HPLC Method

A Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) was a stationary material for the reversed-phase HPLC analysis. Methanol and acetate buffer-based solvent mixture with a 70:30% volume ratio comprised the mobile phase (adjusted to pH 5.6 using OPA). An isocratic elution mode was used with a 1 mL/min flow rate and 20 µL injection volume at 210 nm detection wavelength.

2.6. Analytical Method Validation [18]

Validation shows an analytical technique is strong, trustworthy, and reproducible. Using ICH Q2(R1) recommendations, the following parameters were evaluated.

2.6.1. Specificity

Methanol was used to prepare four types of solutions: blank (diluent only), placebo (excipients), standard (a drug mixture with 20 µg/mL RE and 10 µg/mL VLD), and test (a tablet sample with 20 µg/mL RE and 10 µg/mL VLD). The solutions were analyzed using both a UV-Vis spectrophotometer and an HPLC system to determine if they could accurately measure the analyte even in the presence of interfering agents. The peak purity index of the separated analytes was determined by analyzing the RE and VLD peak profiles in chromatograms produced by injecting a sample solution with 20 µg/mL RE and 10 µg/mL VLD.

2.6.2. Linearity and Range

Validation for linearity requires the preparation and analysis of a set of several independently prepared solutions. To evaluate the linearity of the method, mixed standard solutions of RE and VLD were prepared by diluting stock standard solution with the mobile phase to obtain different concentrations of RE (10, 20, 30, 40, 50, and 60 µg/mL) and VLD (5, 10, 15, 20, 25, and 30 µg/mL), corresponding to 50%, 100%, 150%, 250%, and 300% of target concentration, respectively. Using a UV-Vis spectrophotometer and HPLC equipment, these solutions were analyzed using n=3 determinations.

2.6.3. Accuracy

The closeness of the observed values to the standard value expresses the accuracy of an analytical technique. For the accuracy studies, RE and VLD target concentrations of 20 µg/mL and 10 µg/mL, respectively, were chosen. Three concentration levels (80%, 100%, and 120%) were analyzed using a UV-

Vis spectrophotometer and HPLC system for both drugs. The percent recovery and relative standard deviation (RSD) were calculated.

2.6.4. Precision

Various sample solutions of RE (10, 20, and 30 µg/mL) and VLD (5, 10, and 15 µg/mL) were utilized to evaluate intermediate precision experiments (repeated results on the next day) and repeatability studies (repeated results on the same day). The drug recovery percentage was calculated for the selected levels. Using % RSD and standard deviation (SD), precision experiments were assessed.

2.6.5. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The following equations were used to calculate LOD (1) and LOQ (2).

$$LoD = 3.3 \times \frac{\delta}{s} \ldots . (1) \; ; LoQ = 10 \times \frac{\delta}{s} \ldots . (2)$$

where,
δ= standard deviation
s= slope

2.6.6. System Suitability Studies for Reversed Phase HPLC Method

A standard solution comprising RE (20 µg/mL) and VLD (10 µg/mL) was injected six times into the system to get the suitable settings (retention time, tailing factor, resolution and theoretical plate count).

2.6.7. Robustness Studies for HPLC Method

The method's robustness was tested by changing the method's settings, like the mobile phase composition, pump flow rate, pH, wavelength, and temperature. Three duplicates were made for the target concentration (20 µg/mL RE and 10 µg/mL VLD), and the percent RSD was calculated.

2.7. Assay of Marketed Formulation

Twenty Remo V tablets (label claim to contain 100 mg RE and 50 mg VLD) were taken and crushed. A volumetric flask (100 mL) was filled with a powder equivalent to the label's claim. It was dissolved by adding 50 mL of methanol and sonicating it for 20 minutes. Methanol was used to bring the volume up to par. The resulting solution was taken out and transferred to a volumetric flask (100 mL) where it was diluted with methanol to the required concentrations of 20 µg/mL for RE and 10 µg/mL for VLD. A devised procedure was used to assess the result.

3. Results and discussion

3.1. Development and Optimization of Analytical Methods

The present research aimed to develop UV spectrophotometric and HPLC-PDA methods for estimating RE and VLD in a combined dosage form. The individual drug solutions were scanned between 200 and 400 nm (UV region) at various concentrations to create the UV spectroscopic approach. The wavelength maxima were 232 and 217 nm for the RE and VLD, respectively. The RE and VLD showed maximum absorbance at 232 and 217 nm. Further, a simultaneous equation, Q-absorbance, and derivative spectroscopic method were developed for the said combination.

For the HPLC method development, research publications focusing on analytical methodologies for the individual quantification of RE and VLD were thoroughly examined. Most of these papers proposed using a lower detection wavelength, such as 210 nm, and a mix of organic solvent systems (e.g., acetonitrile, methanol) with water or buffers in the pH range of 3 to 7. The ideal wavelength of 210 nm was chosen to

get a good detector response. Based on early experiments and a literature review, the reversed-phase C18 column was chosen considering its lower polar nature than C4 and C8. Generally, reverse-phase chromatography is recommended for ionic and moderate to polar compounds. Because RE and VLD are basic compounds, the mobile phase pH had to be lower than their pKa (pKa of RE= 12.2 and VLD= 14.71) to improve the separation and resolution of the two peaks. At mid pH, protonated bases typically interact with ionized silanols on the silica of the HPLC column, causing peak asymmetry and technique variability. At acidic pH levels, unprotonated acid can compete with protonated silanols for hydrogen ions.

As a result, when silanols are fully protonated, the silanol-base interaction is negligible, resulting in peaks with excellent resolution and a low tailing factor. The initial trials aimed to select acceptable and optimal chromatographic conditions for separating the specified chemicals from their combination. The primary goal of LC technique development is to obtain acceptable analyte resolution with well-defined symmetrical peaks in a reasonable amount of time. As a result, various tests were carried out utilizing a C18 column to optimize the mobile phases. It distinguished between the RE and VLD peaks and was proven stable at the optimal pH (1.5–9.0) and ionic strength. The parameters that resulted in a satisfactory resolution between the two analyte peaks were chosen as optimal. The best view for this mixture's separation came from a linear isocratic elution of the mobile phase system composed of methanol at a ratio of 30:70 v/v and an acetate buffer solution adjusted to a pH of 5.6 using orthophosphoric acid. The trials used a flow rate of 1 mL/min, a 20 µL injection volume, and a temperature of 25 °C due to the column's internal diameter (4.6 mm). The aqueous mobile phase component (acetate buffer pH 5.6) can be deemed necessary to separate this mixture. The aqueous component's pH was adjusted by experimenting with different pH values [3-5.6], and we found that doing so speeds up the elution of the RE peak while having little effect on the VLD peak. As a result, pH 5.6 was the best compromise between moderate retention times and good peak separation. For example, water and phosphate buffer produced broad-tailed peaks, alterations in retention duration, and poor resolution, notably for VLD. According to optimization attempts, using methanol as an organic phase resulted in crisp symmetric peaks with sufficient resolution.

3.2. System Suitability Test

Numerous factors, such as the theoretical plate count, the tailing factor, and the retention time (Rt), were examined to assess the system suitability of the HPLC method (Table 2). The percentage RSD of the peak area was within the permissible range, as was the retention time. The number of theoretical plates exceeded 2000. All these results proved the HPLC system's suitability.

Table 2. System suitability parameters of HPLC method

Parameters	RE	VLD	Limits
Number of theoretical plates	6143.92±18.0633	2241.76±18.0014	NLT 2000
Retention time (min)	14.341±0.0200	3.438±0.0095	-
Resolution	21.354±0.015		NLT 2.0
Tailing factor (TF)	1.04±0.0050	1.37±0.0050	NMT 2.0

3.3. Linearity

The concentrations and the responses of the HPLC and UV techniques were discovered to be linearly related. Table 3 presents the results of the regression analysis (n=3). Using information from the calibration curve's linear regression equation, the LOD and LOQ were calculated using mean values. The LOD and LOQ values demonstrated the analytical techniques' excellent sensitivity.

Table 3. Calibration curve results, limit of detection and limit of quantitation (n=3)

Parameters	UV spectroscopy		HPLC	
	RE	**VLD**	**RE**	**VLD**
Linear dynamic range (µg/mL)	10-60	5-30	10-50	5-25
Slope	0.0259 ± 0.0012	0.0458 ± 0.0011	27517 ± 527.99	12597 ± 361.48
Intercept	0.021 ± 0.0011	0.0035 ± 0.0012	53239 ± 10015	3758.5 ± 8896
Correlation coefficient	0.9981 ± 0.0005	0.9987 ± 0.0004	0.9934 ± 0.0046	0.9954 ± 0.0055
LOD (µg/mL)	4.85	2.54	3.54	1.05
LOQ (µg/mL)	9.29	4.69	8.89	3.90

3.4. Specificity

The spectra and chromatograms obtained from various solutions using the UV and HPLC methods were observed for the specificity studies. The spectra of the blank (a), placebo (b), standard (c), and sample (d) of RE and VLD are shown in Figure 3. A straight baseline at the wavelength maxima of either drug showed no influence of excipients.

Figure 4 displays the HPLC Chromatograms of the RE and VLD samples, the placebo, the standard, and the blank. The technique's specificity was ascertained by examining the chromatograms concerning the retention times of RE and VLD. There was no indication that the excipients affected the retention times of either drug, and there was no significant noise in the baseline. Peak purity indices of 0.99 and 0.99 for RE and VLD, respectively, neither RE nor VLD found any contaminants (Figure 5).

Thus, it was established that no comigrating or coeluting contaminants were responsible for the peak's reaction. These results show that both methods are specific and may be used to determine RE and VLD in pharmaceutical formulations.

3.5. Accuracy Studies

This parameter demonstrates the accuracy estimation study using the target concentration standard solution spiked at 80%, 100%, and 120% against 100%. The UV and HPLC methods' standard operating protocols were used to compute the recovery. Table 4 displays the numbers. All the observed results fell within the expected range. Therefore, the suggested procedures for assay RE and VLD in a combined dosage form can be used regularly.

Table 4. Accuracy studies (n=3)

Drug	UV spectroscopy					
	Mean Recovery			**% RSD**		
	80 %	**100 %**	**120 %**	**80 %**	**100 %**	**120 %**
RE	102.6	100.21	103.44	1.66	0.79	0.88
VLD	100.38	100.22	99.06	0.84	0.85	0.51
Drug	HPLC					
	Mean Recovery			% RSD		
	80 %	100 %	120 %	80 %	100 %	120 %
RE	102.26	102.00	101.00	0.10	0.39	0.44
VLD	100.75	103.10	103.14	0.61	0.22	0.30

3.6. Precision Studies

The developed methods' precision findings were satisfactory and compliant with ICH requirements. Table 5 presents the findings. The percent recovery was calculated by testing the produced samples at 50, 100, and 150 percent of the target concentration. The intra-day and inter-day analyses showed that the %

RSD was less than 2.0. Because of this, the system appropriateness of the suggested approaches was good, as was the system's accuracy.

Table 5. Precision studies (n=3)

Method	Drug	Concentration (µg/mL)	Intraday (% RSD)	Interday (% RSD)
UV spectroscopy	RE	10	1.35	1.04
		20	1.68	1.50
		30	1.50	1.17
	VLD	5	1.48	1.94
		10	1.52	1.09
		15	1.27	1.82
HPLC	RE	10	0.19	1.48
		20	1.06	0.68
		30	0.64	0.90
	VLD	5	1.16	1.51
		10	0.58	1.26
		15	0.52	0.65

3.7. Robustness Studies

The robustness of the HPLC method was tested by infusing a solution with a known concentration into the method's enhanced conditions with a small modification. Table 6 summarizes the data and demonstrates that the robustness values are good and that there was little variance in the results, demonstrating that the approach was resistant to deliberate modifications.

Table 6. Robustness studies (n=3)

Parameters	Variation	HPLC	
		RE (% RSD)	VLD (% RSD)
Temperature (°C)	20 25 30	0.45	0.77
Wavelength (nm)	208 210 212	0.66	0.79
Methanol volume (mL)	28:72 30:70 32:68	0.51	1.26
pH	5.4 5.6 5.8	0.68	0.89
Flow rate (mL/min.)	0.8 1.0 1.2	1.05	1.11

3.8. Assay of Marketed Formulation

The established chromatographic and spectrophotometric procedures were used to analyze RE and VLD in Remo V (Table 7). At a confidence level of 0.05, an ANOVA test showed a statistically significant difference between the samples' findings from the various procedures. Chromatographic analysis was the

most sensitive and selective method, which could analyze and quantify RE and VLD in biological matrices. However, the cost and time of the analysis prevent us from being paid. In addition to being simple to use and producing less residue, the spectrophotometric approach is also less expensive and takes less time to analyze. Uncer

Table 7. Assay of marketed tablet formulation and uncertainty estimation

Method	Drug	Label claim (mg)	Amount recovered (mg)	Assay (%)
UV spectroscopy	RE	100	98.66	98.66
	VLD	50	48.33	96.66
HPLC	RE	100	100.89	100.89
	VLD	50	49.97	99.94

3.9. Unceratinty Assement

Based on the EURACHEM CITAC Guide and related literature [18-19] the sources of uncertainties were determined as purity of standards, calibration curve, recovery, and precision of the applied methods. Uncertianty budget of the methods were summarized in Table 8 below.

Table 8. Uncertainty budget of the remogliflozin etabonate and vildagliptin in a tablet formulations

Uncertainty	RE		VLD	
	UV method	HPLC method	UV method	HPLC method
Standard *u*	3.53	3.71	3.41	4.02
Expanded U % (k=2, 95 %)	7.05	7.43	6.81	8.04

4. Conclusions

For the determination of RE and VLD in fixed-dose tablet formulations, the UV spectroscopic and HPLC method was developed and validated. They were found adequate to quantify RE and VLD in a fixed dose tablet formulation; the chromatographic and spectrophotometric methods presented the most reliable results. These methods were validated per ICH guidelines, and the findings were confirmed within acceptable ranges. These techniques can be successfully used in quality control analyses to measure and identify RE and VLD in pharmaceutical products since they are quick and easy.

Acknowledgements

The authors gratefully acknowledge the gifts of RE and VLD from Torrent Pharmaceuticals in Ahmedabad, India, and Aalidhra Pharmachem Pvt Ltd. in Vadodara, India.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

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