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Determination of Novel Promising Combination of Linagliptin and Pioglitazone HCl in Bulk and Laboratory Synthetic Mixture by Earth-Friendly Three Spectrophotometric Methods

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Abstract

The combination between peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists and dipeptidyl peptidase-IV (DPP-IV) inhibitors is proven to be a more effective management of type-II diabetes mellitus (T2DM), compared to either each as single therapy. One of the novelist promising combination is linagliptin (LIN) and pioglitazone HCl (PIO) within clinical trials phase-III. Three simple, precise, accurate, reliable, and earth-friendly spectrophotometric techniques were defined and validated for resolving this novel pharmaceutical combination within laboratory synthetic mixtures and bulk form without the need of previous separation. Method-I was first-derivative (D^1) spectrophotometry based on estimating LIN at 247.80 nm absorbance (PIO's zero-crossing point) and PIO at 258.40 nm absorbance (LIN's zero-crossing point) exhibiting mean percentage recoveries of 100.55% and 99.40%, respectively. Method-II was area under curve (AUC) technique involving area measurements at two designated wavelength ranges; 292-305 nm and 265-279 nm for respective LIN and PIO determination and with respective mean percentage recoveries 99.65% and 99.67%. Method-III was isosbestic point spectrophotometry where collective concentrations of both analytes were estimated at isosbestic point. 273.05 nm. Concerning PIO, the concentrations were furnished through simple deduction of LIN concentration, previously determined by AUC technique, and a mean % recovery of 99.88 % was obtained. All three proposed techniques were linear over concentration range 6-30 µg/mL and 5-50 µg/mL for LIN and PIO, respectively. Moreover, such techniques were validated as through the laboratory-prepared mixtures as refer to the International Council for Harmonization (ICH) guidelines.

Keywords: Linagliptin; Pioglitazone HCl; Earth-friendly; First-derivative spectrophotometry; Area Under Curve; Isosbestic point.

1. Introduction

Linagliptin (LIN), chemically identified as (*R*)-7-but-2-ynyl-8-(3-amino piperidinyl)-1-(4-methyl-quinazolin-2-ylmethyl)-3-methyl-3,7-dihydropurine -2,6-dione (**Fig. 1a**), exhibits dipeptidyl peptidase-4 (DPP-4) inhibition activity and a distinct xanthine-based scaffold. Blockage of such enzyme prevents the destruction of incretin hormones: gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) triggering post-prandial insulin secretion and decrease that of glucagon for further blood glucose level reduction (Del Prato et al. 2011). Pioglitazone HCl (PIO), chemically entitled 5-(*p*-[β-(5-ethylpyridin-2yl) ethoxy] benzyl)-1,3-thiazolidine-2,4-dione monohydrochloride (**Fig. 1b**), is a glitazone drug class member exerting its pharmacological action mainly via enhancing insulin sensitivity and promoting blood glucose uptake. Such hypoglycemic action is primarily via selective interaction with the peroxisome proliferator-activated receptor-gamma (PPAR-γ) controlling the glucose and lipid metabolism within muscles, liver, and adipose tissues (Aronoff et al. 2000).

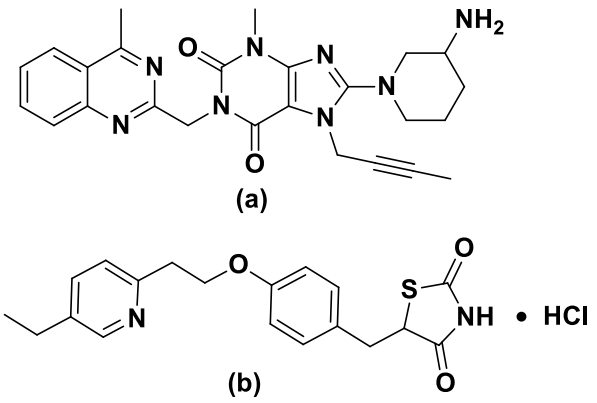

Fig. 1. Chemical structures of investigated analytes; (a) LIN; (b) PIO.

Literature survey revealed a limited number of reported analytical technique for individual quantification of LIN. These methods include spectrophotometry (Badugu et al. 2012, Banik et al. 2013), spectrofluorimetry (El-Bagary et al. 2014), UPLC (Dubey et al. 2014, Ayoub et al. 2015), HPLC-UV (Badugu et al. 2012) and LC-MS (Begum et al. 2014, Shantikumar et al. 2015, Rao et al. 2016) analytical techniques. On the other hand, several analytical techniques have been developed for PIO individual quantification including; HPLC (Jedlička et al. 2004, Souri et al. 2008, Hussein et al. 2015) UV-spectrophotometry (Mahadik et al. 2012). Recently it was found that the combination between DPP-4 inhibitor and PPAR-γ agonist is proven to be more effective therapy for type-II diabetes mellitus (T2DM) compared to either each as single therapy (Rosenstock et al. 2006, Bosi et al. 2007, Mikhail et al. 2008). Only one combination of these two classes has been approved for T2DM treatment, PIO in combination with alogliptin benzoate (Rosenstock et al. 2006). On the other hand, the novelist promising combination between LIN and PIO is currently undergoing clinical trials, phase-III study (ClinicalTrials.gov identifier #: NCT01183013) (Gomis et al. 2011, Nauck et al. 2016). Notably, the latter pharmaceutical combination was well tolerated and furnished clinically significant improvements within the euglycemic control most likely related to the complementary mechanistic aspects of both drugs. Accordingly, combining LIN and PIO can offer valuable preliminary management option for such a

chronic disease, particularly for patients being intolerant or contraindicated to metformin as in case of renal impairment. For simultaneous analysis of LIN and PIO, only one reported method has been developed being depending on dual wavelength spectrophotometry (Patel at al. 2017). Nevertheless, this reported method suffered from many drawbacks where firstly, it was not either eco-friendly for using methanol as solvent or properly validated as per the International Council for Harmonization (ICH) guidelines making it unreliable for routine quality control analysis. Secondly, the reported technique investigated only one ratio of the mixture. Finally, the authors claimed that LIN can be directly estimated from its zero-order spectrum at 295.88 nm, however, this was practically inaccurate since PIO possessed little but not negligible absorbance at such wavelength allowing false positive % recovery of LIN at this wavelength. Therefore, the aim of the present study is to develop, optimize, as well as validate a fast, simple, sensitive, eco-friendly, accurate, and inexpensive spectrophotometric techniques for simultaneous estimation of LIN and PIO. The proposed area under curve, isosbestic point, and first derivative (D^1) spectrophotometric technique provided no prior separation and allowed minimal data processing through simple mathematical calculations. Moreover, the three furnished techniques were validated based on the ICH reported guidelines (Q2(R1) 2005).

2. Experimental

2.1. Materials and reagents

Authentic reference standard of PIO (99.76 %w/w) or LIN (> 99.00 %w/w) was provided as gift samples from SigmaTM Pharmaceutical Industries, Quesna, Egypt or purchased from Wuhan-Vanz™ Pharmaceutical Inc., Wuhan, China. Hydrochloric acid (37 %v/v Analytical grade, Merck™, Germany) was utilized.

2.2. Instrumentation

Schimadzu-UV1800® (Schimadzu™, Japan) the UV/VIS double-beamed spectrophotometer supplied with 10 mm quartz-cell was adopted for the analytical purposes. Recording the absorption spectra was performed at 200-400 nm wavelength range, while data acquisition and processing were achieved by UV-Probe® V2.33 software (Schimadzu™, Japan).

2.3. Stock standard and working solutions

Stock standard solutions (1000 µg/mL) and working solutions (100 µg/mL) of each analyte, LIN and PIO, were constituted using 0.1N hydrochloric acid as an earth-friendly aqueous non-organic diluent. Sonication for 15 min was performed to ensure complete dissolution. Both stock and working solutions were found to be stable for a week when being refrigerated at 4 °C.

2.4. Standard calibration graphs

Several aliquots of LIN working solutions were diluted via 0.1N hydrochloric acid diluent for furnishing several LIN concentrations within the range 6-30 µg/mL. Concerning PIO, aliquots of respective working solution were processed as above for obtaining several concentrations in the range 5-50 µg/mL. Zero order absorption spectra were recorded against 0.1N hydrochloric acid as negative control sample over 200-400 nm wavelength range.

Method-I: First derivative (D^1) spectrophotometric technique

The D^1-spectra for LIN (6-30 µg/mL) at delta lambda ($\Delta\lambda$)=4 and scaling factor (SF)=1.0, were estimated and D^1-amplitude values, at 247.8 nm PIO Zero-crossing, were plotted versus relative concentrations furnishing the standard calibration graph of LIN. For constructing the PIO standard calibration graph, same previous steps were followed for PIO (5-50 µg/mL) D^1-spectra, except, the D^1-amplitude values were plotted at 258.4 nm (LIN zero-crossing). Regression equations were then computed using the Microsoft office Excel® 2019 (Microsoft™, USA).

Method-II: Area under curve (AUC) technique

Standard calibration graphs were constructed through plotting the analyte concentrations against their corresponding AUC of zero-order spectra within 292-305 nm and 265-279 nm wavelength ranges for LIN and PIO, respectively. Regression equations were then deduced as in method I.

Method-III: Isosbestic point (ISP) technique

Standard calibration graphs were constructed through plotting the analyte concentrations against their corresponding zero-order absorbance amplitudes at the ISP (273.05 nm). Regression equations were then estimated as in method I.

2.5. Laboratory synthetic mixtures

Three laboratory-prepared synthetic binary mixtures were constructed at different LIN:PIO ratios, 1:3, 1:6, and 2:1 through diluting respective aliquots of LIN and PIO working solutions with 0.1N Hydrochloric acid. Concentrations (µg/mL) of LIN:PIO were set at 10:30, 6:36 and 20:10 for binary mixture-I, -II, and -III, respectively. Calculations, regarding both precision and accuracy, were estimated via these binary mixtures.

3. Results and discussion

Comparing to the more sophisticated and expensive HPLC methods, spectrophotometry still offers more efficient, high simple, cost efficient, and time saving techniques for routine analysis and quality control of commercial pharmaceuticals. Nevertheless, direct UV-determination of active compounds within binary mixtures can be just feasible when both agents are totally resolved at absorbance maxima (λ_{max}).

LIN and PIO are a promising novel combination for the management of T2DM, and thus, there is an urgent demand for finding efficient technique(s) concerning their simultaneous determination within respective binary mixtures.

As illustrated in **Fig. 2**, the zero-order absorption spectra of PIO (30 µg/mL) and LIN (10 µg/mL) within 0.1N hydrochloric acid display severe overlapping within 200-320 nm region. Therefore, simultaneous estimation of LIN/PIO mixture through conventional UV spectrophotometry is hindered. Accordingly, the three spectrophotometric techniques, proposed within this manuscript, exhibit fast, simple, and earth-friendly simultaneous determination of PIO and LIN within their binary mixtures.

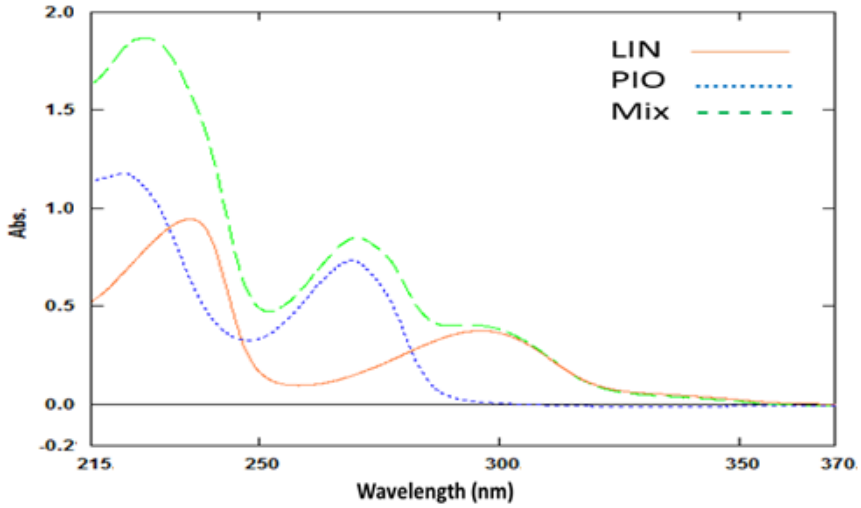

Fig. 2. Overlay of zero-order absorption spectra of 10 µg/mL LIN (—); 30 µg/mL PIO (…..); binary mixture (- - -) comprising 30 µg/mL PIO and 10 µg/mL LIN.

3.1. Method development

Method-I: First derivative spectrophotometric technique

Regarding the of pharmaceutical mixtures showing overlapping spectra, the derivative spectrophotometric techniques, using the zero-crossing approach, have been proven beneficial for simultaneous determinations and matrix background elimination (Mohamed et al. 2006, Abdulameer et al. 2011, Samir et al. 2012). In this report, the LIN/PIO spectra overlap was resolved via the D^1-spectrophotometric technique (**Fig. 3**). Interestingly, the interference was considerably reduced where the D^1-spectra of LIN and PIO shows absorption maxima at 247.8 nm and 258.4 nm, respectively, while the counterpart drugs read zero absorbances. Linear relationships, between the peak amplitudes and corresponding concentrations, were furnished for LIN and PIO at 247.8 nm and 258.4 nm, respectively. Regression equations for D^1-method of LIN and PIO were estimated as $A_{LIN} = -0.0052C_{LIN} - 0.0011$ ($r = 0.9999$) and $A_{PIO} = 0.0008C_{PIO} + 0.0001$ ($r = 0.9999$), respectively. Within such equations, A represents the peak amplitudes; C is for concentrations, while, r is the correlation coefficient.

Method-II: Area under curve technique

The AUC technique represents a simple approach for estimation the concentration of pharmaceutical agents within binary mixtures relying on their respective area of absorption spectrum.

It is applicable when drugs spectra are broad or with no sharp peak (Ghorpade et al. 2010, Abdelrahman et al. 2013, Kant et al. 2016, Mabrouk et al. 2018, Magdy et al. 2019). For simultaneous determination of LIN/PIO using AUC method, different wavelength regions have been investigated within the ranges 292-305 nm and 265-279 nm for LIN and PIO, respectively. Integrating the area between the designated wavelength ranges was performed (**Fig. 4**) and calibrations curves were plotted.

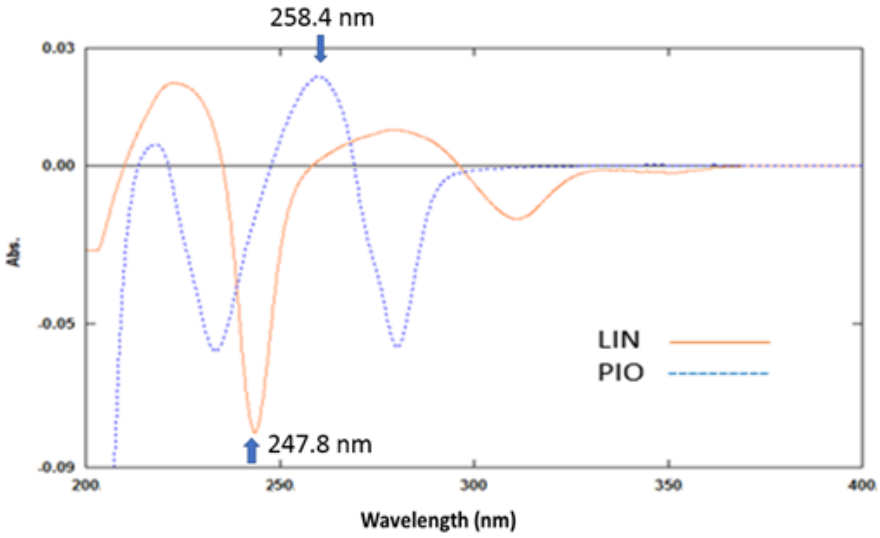

Fig. 3. D[1]-absorption spectra of 10 µg/mL LIN (—); 30 µg.mL^{-1} PIO (…..) in 0.1N hydrochloric acid showing zero-crossing points, Δλ = 4 nm.

Subsequently, two simultaneous equations were constructed, utilizing the priorly integrated areas, where the concentrations of each drug were solved throughout the provided equations:

$$C_{LIN} = \frac{(A_2 * a_{y1}) - (A_1 * a_{y2})}{(a_{x2} * a_{y1}) - (a_{x1} * a_{y2})} \qquad C_{PIO} = \frac{A_2 - (a_{x2} * C_{LIN})}{a_{y2}}$$

Where, C is the concentration in µg%; a_{x1} (-10.21) and a_{x2} (27.44) represent the LIN specific absorbances at 265-279 nm and 292-305 nm, respectively; a_{y1} (37.32) and a_{y2} (-1.17) are the PIO specific absorbances at 265-279 nm and 292-305 nm, respectively.

Finally, A_1 and A_2 are the AUC of binary mixture within designated wavelength ranges 265- 279 nm and 292-305 nm, respectively.

Method-III: Isosbestic point technique

The isosbestic spectrophotometric technique was developed by Erram and colleague, where the spectrum of similarly concentrated drugs under investigation intersect at a point known as isosbestic/isoabsorptivity point (Erram et al. 1994)

Both analytes express equal absorptivity at such assigned point making the binary mixture to act as a singular component possessing similar absorbance as the pure analyte. Thus, the absorbances at ISP is considered as good expression for the collective concentration of both analytes within combination (El-Ghobashy et al. 2010, Riad et al. 2012, Baghdady et al. 2013, EL-Shorbagy et al. 2018). By recording the zero-order absorption spectra of separate analytes and the spectrum of their collective mixture, two ISP s at 255.09 nm and 273.05 nm were detected (**Fig. 5**). Notably, linear correlation, between the absorbance values and corresponding concentrations, was only depicted at the designated ISP (273.05 nm). Following computing the regression equations, findings are corresponding to the total concentrations of LIN and PIO within the LIN/PIO binary mixture. Since the LIN concentration was previously determined using AUC technique (refer Method II), the PIO concentration could be estimated through simple subtraction function.

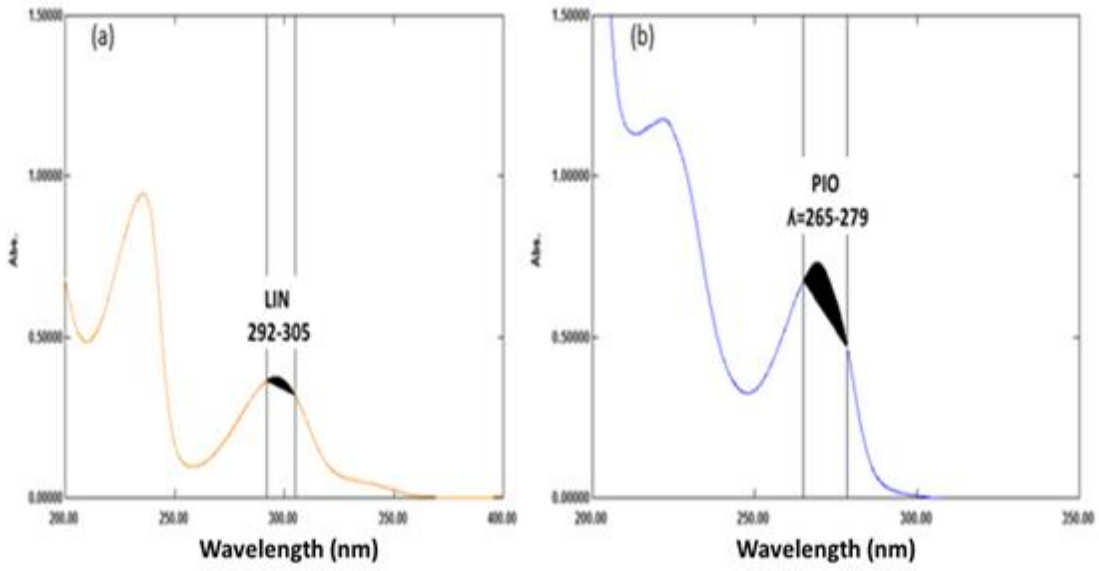

Fig. 4. Zero-order absorption spectra of investigated analytes; (a) LIN (10 µg/mL) exhibiting AUC (292-305 nm) (a); (b) PIO (30 µg/mL) exhibiting AUC (265-279 nm).

3.2. Method optimization

Enhancing the performance of the proposed D^1, AUC, and ISP spectrophotometric techniques was achieved through optimizing several parameters. Optimum diluent was chosen through utilizing several solvent systems either aqueous or organic. Both analytes were best soluble in methanol and 0.1N hydrochloric acid as compared to other solvent, however, 0.1N hydrochloric acid was chosen for furnishing superior selectivity and sensitivity as well as being cheaper and much greener. Optimizing the D^1-derivatization performance was done through several Δλ values and SF being at 2, 3 & 4 nm and from 1-to-5, respectively. Notably, the Δλ at 4 nm and SF set at 1.0 were the most appropriate parameters upon which maximum peak height was obtained with good resolution and minimum noise especially for small concentrations. Assigning a specific wavelength region for constructing an AUC technique can greatly impact the analytical parameters, including; intercept, slope, and correlation coefficient. Thus, several wavelength regions were examined, and optimum findings were depicted throughout the 292-305 nm and 265-279 nm ranges for LIN and PIO, respectively. Such adopted wavelength ranges were assigned based on drug's achieved sensitivity and repeated observations for obtaining good linearity between AUC and respective concentrations. Obtaining such favored linearity was beneficial for better differentiation between drugs as well as providing good selectivity and % recovery. Regarding the ISP technique, there were two isosbestic points, at 255.09 nm and 273.05 nm (**Fig. 5**), where both drugs have equal absorptivity. Nevertheless, the isosbestic (at 273.05 nm) was found optimum for calculations on the basis of good linearity and % recovery.

3.3. Method Validation

Validating the capacity of the proposed spectrophotometric techniques to simultaneous estimate LIN and PIO within respective binary

Table 1. Statistical parameters and performance findings for LIN/PIO binary mixture estimation via the proposed spectrophotometric techniques

Parameters	D^1 technique		AUC technique		ISP technique	
	LIN at 247.8 nm	PIO at 258.4 nm	LIN area (292-305 nm)	PIO area (265-279 nm)	LIN at 273.05 nm	PIO at 273.05 nm
Linearity range (µg/mL)	6-30	5-50	6-30	5-50	6-30	5-50
r	0.9999	0.9999	0.9999	0.9998	0.9998	0.9998
Slope ± SD	-0.0052 ± 3.67E-05	0.0008 ± 2.68E-06	0.0274 ± 0.0003	-0.0102 ± 0.0001	0.0193 ± 0.0002	0.0193 ± 0.0002
Intercept ± SD	-0.0011 ± 0.000668	0.0001 ± 8.15E-05	-0.0024 ± 0.0048	0.0023 ± 0.0019	-0.0178 ± 0.0041	-0.0130 ± 0.0054
LOD (µg/mL)	0.42	0.36	0.58	0.61	0.58	0.86
LOQ (µg/mL)	1.28	1.08	1.75	1.85	1.75	2.59

Table 2. Accuracy assessment data regarding the LIN and PIO estimation via proposed spectrophotometric techniques

		D^1 technique	AUC technique	ISP technique
Drug	**Conc. taken (µg/mL)**	**% recovery* ± RSD**	**% recovery* ± RSD**	**% recovery* ± RSD**
LIN	**6**	98.17 ± 0.44	98.50 ± 0.58	98.51 ± 0.58
	10	102.30 ± 1.62	99.90 ± 1.31	99.94 ± 1.31
	20	102.25 ± 0.23	100.60 ± 0.47	100.02 ± 0.47
PIO	**10**	99.80 ± 0.40	99.6 ± 1.13	100.4 ± 1.04
	30	98.57 ± 0.76	99.17 ± 0.46	99.18 ± 0.61
	36	99.86 ± 0.54	100.28 ± 0.24	101.31 ± 1.26

*Average of three-time determinations per single concentration.

mixtures was conducted as per the ICH guidelines (Q2(R1) 2005) for analyzing various laboratory-prepared mixtures comprising different ratios of the two analytes. The following validation characteristics were addressed.

3.3.1. Linearity and range

Calibration graphs, for five triplicate concentrations of each LIN and PIO, show linearity over the concentration ranges 6-30 µg/mL and 5-50

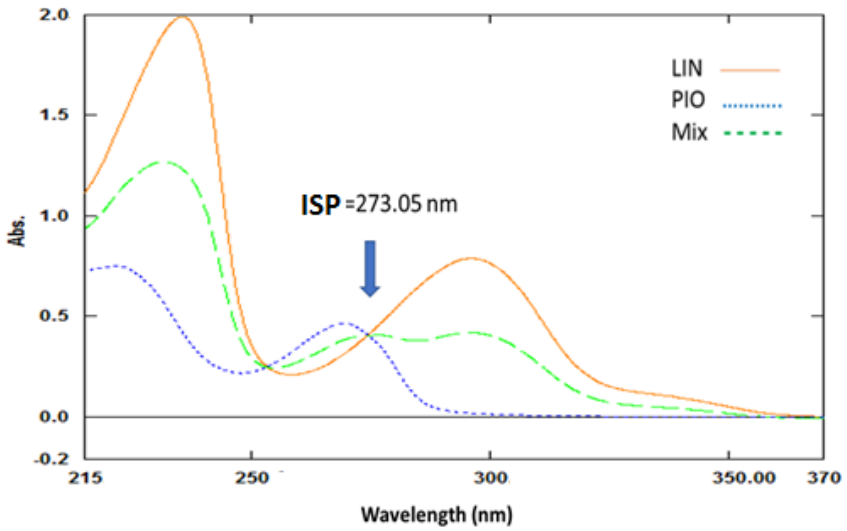

Fig. 5. Zero-order absorption spectra of 20 µg/mL LIN (—); 20 µg/mL PIO (…..) and (1:1) binary mixture comprising 10 µg/mL of each analyte (- - -) using 0.1N hydrochloric acid as blank.

µg/mL for LIN and PIO, respectively. High correlation coefficients values, being near to unity, and negligible intercepts indicate the good linearity of these calibration graphs (**Table 1**).

3.3.2. Detection and quantitation limits

Both minimal limit of quantitation (LOQ) and limit of detection (LOD) indicated the great sensitivity for the three proposed spectrophotometric techniques (**Table 1**). The parameters were estimated theoretically through the following equations: LOQ = 10*(σ/S); LOD = 3.3*(σ/S), where S being the calculated slope value and σ is the standard deviation of responses for triplicate blank measurement. Moreover, the sensitivity of the developed methods was compared to each other. Interestingly, the D^1-technique exhibits the superior sensitivity, for either LIN or PIO, followed by the AUC and then ISP technique (**Table 1**).

3.3.3. Accuracy

Accuracy, of the furnished techniques, was evaluated by analyzing three different concentrations of LIN and PIO, within their respective linearity ranges, and expressed as the % recovery. Findings, within **Table 2**, illustrate the method accuracy for LIN and PIO with satisfactory % recovery being above 96%. The results of accuracy of the three developed spectrophotometric methods were compared to each other. Notably, the D^1-technique is of best accuracy for LIN determination followed by AUC method. On the other hand, the ISP technique was superior of AUC and the least accurate D^1-technique concerning the PIO estimation.

3.3.4. Precision

The precision of the proposed technique was assessed via estimating the intra-day and inter-day precisions through calculating the % recovery ± RSD for the analysis of QC samples in triplicates and on three separate days, respectively (**Tables 3 and 4**). The obtained results reflect the reliability of the proposed methods during its routine application for simultaneous LIN/PIO analysis within their binary mixture.

Table 3. Intra-day precision assessment data regarding the LIN and PIO analysis

		D[1] technique	AUC technique	ISP technique
Drug	**Conc. taken (µg/mL)**	**% recovery* ± RSD**	**% recovery* ± RSD**	**% recovery* ± RSD**
LIN	**6**	99.83 ± **1**.44	90.17 ± 1.58	96.17 ± 0.32
	10	100.80 ± 2.62	97.7 ± 0.31	95.90 ± 1.33
	20	101.50 ± 4.23	101.65 ± 0.47	100.66 ± 0.55
PIO	**10**	97.40 ± 0.60	94.39 ± 1.3**0**	100.90 ± 1.44
	30	97.43 ± 0.86	98.55 ± 0.33	99.00 ± 1.61
	36	97.50 ± 0.99	100.36 ± 0.44	102.14 ± 0.26

*Average of three-time determinations per single concentration.

Table 4. Inter-day precision assessment data regarding the LIN and PIO analysis

		D[1] technique	AUC technique	ISP technique
Drug	**Conc. taken (µg/mL)**	**% recovery* ± RSD**	**% recovery* ± RSD**	**% recovery* ± RSD**
LIN	**6**	99.68 ± 5.98	99.33 ± 1.43	96.48 ± 1.43
	10	100.31 ± 10.03	99.69 ± 1.16	99.97 ± 1.16
	20	101.00 ± 20.20	101.15 ± 0.69	102.11 ± 0.69
PIO	**10**	99.35 ± 9.95	99.66 ± 1.00	99.04 ± 0.88
	30	98.93 ± 29.68	99.97 ± 0.95	99.57 ± 0.41
	36	99.56 ± 35.84	100.31 ± 0.10	103.24 ± 0.66

*Average of three-time determinations per single concentration.

4. Conclusion and future work

The current study describes three simple spectrophotometric techniques applied for the simultaneous estimation of a novel promising anti-diabetic combination (LIN and PIO) without the need for prior separation, while as providing minimal data procession through simple mathematical calculations. Such techniques are precise, sensitive, cost-effective, accurate, and Earth-friendly which are advantageous over the other reported technique for same mixture estimation. Accordingly, the suggested techniques are proven beneficial being more suitable and being reliable for the routine quality control analysis of LIN and PIO binary mixtures. Future work will consider the evaluation of the proposed techniques in presence of pharmaceutical excipients as well as investigating the mutual impact of combined LIN and PIO on each their pharmacokinetic and drug-drug interaction.

Conflict of interest

The authors declare a no conflict of interest nor receive any specific grant from any funding agencies in the public, commercial, or even not-for-profit sectors.

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