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Green analytical chemistry and quality by design: A combined approach towards simultaneous determination of Letrozole with its co-administered Zoledronic Acid for cancer patients

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ABSTRACT

Nowadays, breast cancer is the most affecting and globally diagnosed malignancy among women, yet Letrozole (LTZ) was considered the first-line treatment as hormonal anticancer drug. Unfortunately, LTZ develops osteoporosis as a main side effect which was overcome by using the co-administered; Zoledronic Acid (ZDA). Thus, there was a crucial need for this simultaneous quantification innovation, especially there were no any previously reported methods regarding both drugs together. In this study, an integrated framework was conducted between the experimental *analytical quality-by-design* (AQbD) approach and green analytical chemistry (GAC), emerging sensitive and robust RP-HPLC method. *Box-Behnken Design* was the developed model for optimizing an isocratic chromatographic separation on C_{18} Equisil® ODS (4.6 × 250 mm, 5.0 μm) column at ambient temperature, using the mobile phase of 0.1 % aqueous trifluroacetic acid (pH 2.8): acetonitrile (54.5:45.5, v/v), at 1.0 mL/min flow rate with PDA detection at 254.0 nm and 210.0 nm for LTZ and ZDA, respectively. Model statistical and residual plots analysis was significant and normally distributed. Method was fully validated as per ICH guidelines, where good linearity was 0.20–10.00 μg/mL for both drugs in presence of Tadalafil (TDF) as an internal standard, obtaining adequate correlation coefficients **(r)** values. Calculated LOD results were 0.058 and 0.040 μg/mL while calculated LOQ results were 0.175 and 0.122 μg/mL for LTZ and ZDA, respectively. The proposed method was effectively applied on bulk, pharmaceutical dosage forms, and spiked human plasma. Statistical comparison of the anticipated results with the reported ones was performed. Greenness assessment was evaluated using Green Analytical Procedure Index (GAPI) and Analytical Greenness (AGREE) tools; where superiority results were achieved relative to other reported methods. Finally, an EVG method evaluation tool was assessed, and the attained results were represented through its radar chart.

1. Introduction

Breast cancer is the most affecting and globally diagnosed malignancy among women, and its complications may lead to death. There are several pathways for the treatment of breast cancer such as surgery, radiation, chemotherapy, and hormonal therapy, while recent studies prove the efficacy of hormonal treatment as a first-line treatment [1].

Letrozole (LTZ); chemically known as [4,4-(1H-1,2,4-Triazole-1-yl methylene) bis-benzonitrile)]; (Fig. 1**a**) is an adjuvant, potent, non-steroidal, third-generation aromatase inhibitor drug [2]. LTZ plays a vital role in hormonally-responsive breast cancer regarding women's metastatic and non-metastatic cases. It selectively inhibits the conversion of testosterone into estradiol (estrogen precursor), reducing circulating estrogen plasma levels [3].

Osteoporosis or osteopenia is a well-common developed adverse effect, regarding LTZ therapy intake. This returns to the loss in bone mineral density accompanied by estrogen decrease in plasma levels, although, clinically bone metastasis whether benign or malignant may arise. Thus, there is a crucial need for a co-administered drug, Zoledronic Acid, to overcome cases' severity [4].

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Zoledronic Acid (ZDA); chemically known as [(1-hydroxy-2-imidazol-1-yl-1-phosphonoethyl) phosphonic acid]; (Fig. 1**b**) is a newly third generation bisphosphonate drug [2]. ZDA inhibits the resorption of osteoclasts, leading to a skeletal stability increase against fractures, and prevents bone metastasis [5]. Systematic anti-cancerous effect of ZDA was mainly observed in postmenopausal women with no precise mechanism known yet [5].

Several methods were reported for LTZ analysis including spectroscopic [6], chromatographic [7–18], and electrochemical [19–21], while few methods were reported for ZDA analysis including spectroscopic [22], chromatographic [23–29], and electrochemical [30]. Fortunately, no reported methods were found for the simultaneous determination of both drugs.

Green Chemistry was firstly mentioned by Anastas [31]; including four important parameters that could only be applied for analytical chemistry goals; which are waste prevention, safer solvents, energy efficiency, and derivatization reduction. Subsequently, the green analytical chemistry (GAC) agreement was introduced with its twelve approaches; the four previously mentioned, and eight others were newly added; including direct analysis, decreasing sample size, in situ measurements, automated methods, real-time analysis, reagents reduction, renewable reagents source, and operator safety. The correct application of GAC ensures lots of benefits in different aspects of sustainability and environmental protection. Therefore, it is highly important to evaluate any chemical analysis process for its numerous steps and complexity; guaranteeing that it follows this agreement [32].

Traditional HPLC method development or one-factor-at-a-time approach mainly depends on trial and error approach, where the separation is controlled by several variables such as column type, column temperature, pH of mobile phase, buffer type, flow rate, detection wavelength, … etc [33]. Consequently, large number of experimental runs are performed through continuous changing one chromatographic parameter till obtaining a perfect desired peak, leading to time consumption, much effort, high budget, and partially interpreted results. Yet, this could be controlled by using the advanced ***Analytical Quality-by-Design*** **(AQbD)** approach ***(includes Doehlert Design, Box-Behnken Design, and Central-Composite Design)***.

In the last decade the AQbD approach has been used in several applications in the analytical field [33]. The first AQbD step regarding method development process is the ***Risk Assessment Studies'*** **(RASs)**, where it identifies mainly the critical method parameters (CMPs) and factors that have impact on the critical quality attributes (CQAs) such as short time analysis, fine peak symmetry, large number of theoretical plates, and greenness of method. RASs examine various factors that control the target method quality profile [34].

Box-Behnken Design (BBD) was proposed since 1960s and became the most valuable approach for multivariate conditions' optimization. It is based on three-factor analysis combined in blocks, excluding the results of combined variables at their extremities (highest and lowest levels). BBD has superior advantages over other designs' approaches such as reducing the total number of experimental runs, enhancing the results' reliability in a unique way, cost efficient, and time saving method optimization. BBD is considered a good methodology for response surface, due to its ability to estimate the quadratic model, detect the model lack of fit; as well it uses blocks and can build sequential designs. Additionally, it shows much more efficient results than the full factorial designs, and slightly efficient results than the central-composite designs [34].

This study aims to develop a newly combined integrated approach between the advantageous of green chemistry and AQbD for the simultaneous chromatographic determination of LTZ and its co-administered drug, ZDA for the first time together. This was achieved by using the distinctive BBD; establishing a novel, green, sensitive, and robust HPLC method according to ICH validation guidelines [35]. Moreover, it is applied on pharmaceutical formulations (Femara® and Zometa®), and on spiked human plasma by using one step protein-precipitation extraction technique. In addition, a comparative study was conducted for the proposed method greenness with the reported literature of each drug separately, using two evaluation tools: Green Analytical Procedure Index (GAPI) and Analytical Greenness (AGREE).

2. Experimental

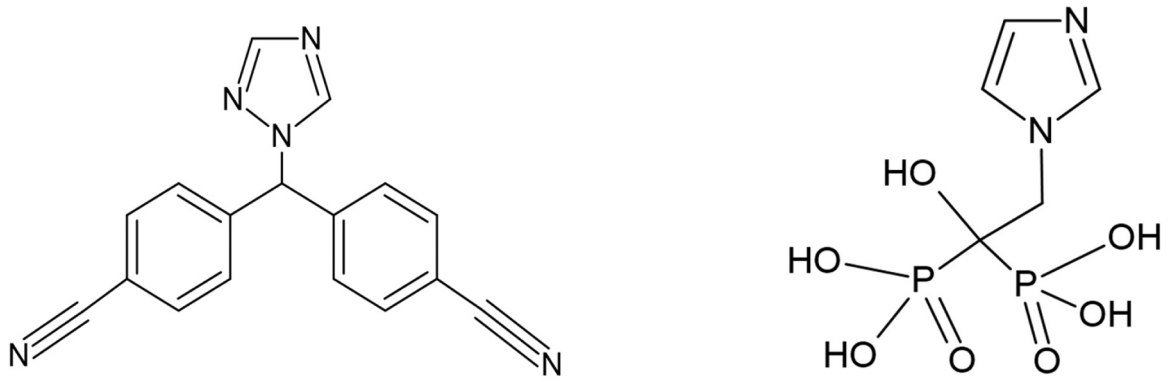
2.1. Instruments and software programmes

2.1.1. For HPLC method

Chromatographic separation was achieved by using Waters 2690 Alliance HPLC system equipped with a quaternary pump, Waters 996 photodiode array detector, and a vacuum degasser (USA). Method development and quantification were carried on reversed phase C_{18} Equisil® ODS column (4.6 × 250.0 mm, 5.0 µm) (USA). Data processing was done using EMPOWER 3 Software.

Electronic balance (Shimadzu, Japan), magnetic stirrer (Stuart, UK), centrifuge (Zjmzym, China), ultrasonic water bath (Elma, Germany), syringe filters 0.22 µm PTFE membrane (Chromtech, UK) and pH meter (Jenway 3505, UK) were used.

Box-Behnken, a response surface experimental design, and data analysis were interpreted using Design Expert (DX®) software, version 11.1.0.1 Copyright 2021 by Stat-Ease Inc., Minneapolis, MN, USA.



(1a) **(1b)**

Fig. 1. Chemical structure of (1a) Letrozole and (1b) Zoledronic Acid.

2.2. Materials

2.2.1. Pure samples

Letrozole (LTZ) was purchased from Sigma Aldrich (Missouri, USA) and it's purity was determined and found to be 98.40 % ± 1.77 according to *British pharmacopeial* method [2]. Zoledronic Acid (ZDA) was purchased from AmBeed Company (Arlington Hts, USA) and its purity was confirmed to be 98.15 % ± 1.41 according to a reported method [28]. Tadalafil (TDF) and Carbamazepine (CBZ) that were utilized as internal standards were kindly gifted from NODCAR, Egypt, with a certified purity of 99.60 % ± 1.56.

2.2.2. Pharmaceutical formulations

Femara® tablets (Lot no. **SAWJ9)** labeled to contain 2.50 mg of LTZ per tablet and Zometa® injection (Lot no. **SFHT4)** labeled to contain 4.00 mg of ZDA per 5 mL. Both are products of Novartis pharmaceutical company and were purchased from the local Egyptian market.

2.2.3. Chemicals and reagents

All solvents used during analysis were of HPLC grade. Methanol, acetonitrile (ACN), and orthophosphoric acid were purchased from Merck, Germany. Perchloric acid and hexane sulphonic acid were purchased from Scharlau, Germany. Trifluroacetic acid was obtained from Prolabo, China. Analytical Sodium hydroxide grade was bought from Al-Nasr Pharmaceutical Chemical Co., Egypt. Deionized water was obtained from SEDICO Pharmaceuticals Co., Egypt. Human plasma was purchased from VACSERA Company, Egypt.

2.2.4. Standard solutions

Standard stock solutions of 1.00 mg/mL of LTZ and ZDA were separately prepared in solvent mixture of methanol: 0.1 N NaOH (50:50, v/v). Freshly working standard solutions of 100.00 μg/mL were prepared by diluting the stock standard solutions with the same solvent.

2.3. Procedures

2.3.1. Experimental design

Design Expert (DX®) software was used to create a randomized response surface experiment through using ***Box-Behnken Design***. An initial screening analysis was performed for identifying the most effective parameters in the chromatographic separation of this study. Experimental optimization was designed with three CQAs including resolution 1 (RS1), resolution 2 (RS2), and run time (min), as well as three CMPs which are flow rate (mL/min), percentage of organic phase (acetonitrile), and pH of the aqueous phase. The optimized chromatographic conditions were designated in total of fifteen runs. Each trial differed from the other due to interaction effects over the dependent variables. Statistical analyses for all the variables were calculated using one-way analysis of variance (ANOVA) to find out the significant ones and their interactions, as well the desirability function was used to achieve the required final optimization.

2.3.2. Chromatographic conditions

The optimized chromatographic separation was performed using C_{18} Equisil® ODS (4.6 × 250.0 mm, 5.0 μm) column using an isocratic elution of 0.1 % aqueous trifluroacetic acid (pH 2.8): acetonitrile (54.5:45.5, v/v) as a mobile phase. The injection volume was 20.0 μL and the flow rate was 1.0 mL/min at ambient temperature. TDF was used as an internal standard. Detection wavelengths were monitored at 254.0 nm and 210.0 nm for LTZ and ZDA, respectively using photodiode array detector (PDA).

2.4. Method validation

2.4.1. Linearity

In a set of 10-mL volumetric flasks, aliquots of LTZ and ZDA were accurately transferred from their working standard solutions equivalent to 2.00, 5.00, 10.00, 20.00, 40.00, 60.00, 80.00 and 100.00 μg were added and diluted with the mobile phase to get final concentrations of 0.20, 0.50, 1.00, 2.00, 4.00, 6.00, 8.00, 10.00 μg/mL for both drugs. An aliquot of 20 μL of each solution was injected triplicate into the chromatographic system. The previously described conditions were adopted and the chromatograms were monitored at 254.0 nm and 210.0 nm for LTZ and ZDA, respectively. Calibration graphs were plotted relating drugs concentration to peak area response and regression equations were calculated.

2.4.2. LOD and LOQ

LOD and LOQ were calculated by using the equations $\mathbf{LOD = 3.3\,\sigma/S}$ and $\mathbf{LOQ = 10\,\sigma/S}$, whereas $\boldsymbol{\sigma}$ is the standard deviation of the intercept and **S** is the slope of the calibration curve.

2.4.3. Accuracy

Accuracy of the proposed method was evaluated by the analysis of pure samples (LTZ and ZDA) at three different concentration levels (1.50, 3.00, 9.00 μg/mL) in triplicate, where mean recovery% ± SD were calculated.

2.4.4. Precision

Precision was assayed by the analysis of pure samples (LTZ and ZDA) at three different concentration levels (1.50, 3.00, 9.00 μg/mL) using three replicate determinations for each concentration, within one day (intra-day precision), and repeating the same three concentrations on three consecutive days (inter-day precision). Their RSD% (relative standard deviation percent) was calculated.

2.4.5. Robustness

Robustness of the proposed method was assessed through studying the effect of the slight variations, which may arise within the chromatographic conditions, including % of organic phase (± 5 %), and amount of trifluroacetic acid (± 0.025 %).

2.4.6. Selectivity

Selectivity was determined by analyzing LTZ and ZDA in laboratory-prepared mixtures containing both the intact drugs at different proportions.

2.5. Applications of the proposed method

2.5.1. Application on pharmaceutical dosage form

Regarding **LTZ**, ten tablets of Femera® were weighed and finely powdered. An amount equivalent to 2.50 mg was transferred in a 25-mL volumetric flask to which methanol and 0.1 N NaOH (50:50, v/v) were added. After vigorous shaking, volume was completed using the same diluent and then filtered to obtain a solution labeled to contain 100.00 μg/mL LTZ.

Regarding **ZDA**, five mL of Zometa® injection was transferred into a 10-mL volumetric flask, diluted and agitated thoroughly with diluent. 2.5 mL of this solution were filtered using a 0.22 μm membrane filter and were transferred into 10-mL volumetric flask, adjusted to the mark using the same diluent, to obtain a working solution labeled to contain 100.00 μg/mL.

Two μg/mL from each of the obtained solutions, together with TDF (10.00 μg/mL) were analyzed as described under Section 2.3.2 and the concentrations of drugs were calculated from the corresponding regression equations. Moreover, the standard addition technique was performed to check the accuracy of the results, in which different aliquots from the standard solutions were added to constant volume of previously analyzed drug formulation and the recovery % of amount added was calculated from the corresponding regression equations.

2.5.2. Application on spiked human plasma

Different concentrations (0.20, 2.00, 6.00 and 10.00 μg/mL) of LTZ and ZDA working standard solutions were spiked into 100-μL human plasma, in presence of TDF (10.00 μg/mL). Solution was vortexed for 60 s. One step protein-precipitation extraction technique was done by using 300 μL of acetonitrile. The mixture was vortexed for 2 min and then centrifuged at 10,000 rpm and 4 °C for 10 min. Twenty μL from the clear supernatant was injected to HPLC and analysed using the optimized chromatographic conditions. The corresponding drug concentrations were calculated from their corresponding regression parameters.

3. Results and discussion

3.1. Risk assessment and screening studies

The risk assessment strategy is an important component regarding AQbD in reference to the ICH guidelines Q8 and Q9 [36]. As there are lots of factors affecting HPLC method development; those factors may be either significant that should be further studied or non-significant that should be further neglected. Risk assessment investigated many CMPs for the determination of CQAs. Initial trials were performed to develop a suitable method for the separation of the studied co-administered drugs. Different types of stationary phases with varying dimensions were used, including Kromasil® C_8 (4.6 × 250 mm, 5.0 μm) and Equisil® C_{18} (4.6 × 250 mm, 5.0 μm). Preliminary results showed that Equisil® C_{18} adequately separated the studied compounds with good system suitability. Also, the type of mobile phase used affected the separation and analysis time. Various trials with different mobile phase compositions were implemented, including (aqueous phase: mixture of perchloric acid + ortho-phosphoric acid + hexane sulphonic acid in 1000 mL water, or trifluoroacetic acid in 1000 mL water) with (organic phase: ethanol or acetonitrile) **(**Fig. S1**)**. Better separation was achieved by using the mobile phase trifluoroacetic acid with acetonitrile, where this composition was further optimized using BBD. Column ambient temperature was adjusted to ensure high resolution and fine peak shaping. Isocratic mode of elution was kept constant due to its numerous advantages over gradient elution; including its simplicity, economical cost, ease instrumentation, and reduce the requirement for re-equilibration of columns between injections. Also, different ratios of mobile phase composition with different pH and flow rates were trailed and were found to be the most significant among the six screened parameters. So, they were identified as **CMPs** or (independent variables/factors) that have a detectable effect on **CQAs** or (dependent variables/responses) which are RS1 (between ZDA and LTZ), RS2 (between LTZ and TDF), and run time, as well their effects were further studied to get optimum results. On the other hand, column type and column temperature were found to have insignificant effects on the CQAs; accordingly, they remained constant at their best selection in the risk assessment phase; Equisil® C_{18} (4.6 × 250.0 mm, 5.0 μm) column at ambient temperature.

3.2. Method optimization using Box-Behnken AQbD

Box-Behnken Design is an experimental design matrix at three-levels for each independent variable (-1, 0, +1). It was used to optimize and evaluate both linear and quadratic effects, as well as the interaction effects of the studied factors on the responses of interest. The center point (zero level for the three factors) i.e. (0, 0, 0) should be repeated several times through the design to determine pure errors [37].

Therefore, method optimization was achieved by customizing BBD of fifteen runs using the DX® software. For model feeding, all trials were carried out, and their corresponding data for responses were recorded **(**Table S1**)**. In this design, three runs were repeated, which were done at the center point, however, the remaining twelve runs were performed to reduce any biased responses, through the effects of uncontrolled responses. The designated model was established to be linear model for both RS1 and RS2, while quadratic model for run time response. Each main effect, interaction, and quadratic terms was discussed in the later sections. Optimal flow rate was adjusted to be 1.0 mL/min for ensuring good separation, faster elution, and shorter run time.

3.3. Model statistical and residual plots analysis

ANOVA was used mainly for analyzing the models' results statistically as shown in Table S2. Since the responses probability (p-value) is less than 0.05, then the model and terms are considered to be significant. Regarding Table 1, both values for regression models: R^2 (R-squared) and Adj-R^2 (adjusted R-squared) were within the acceptable limits ($R > 0.08$); this helps in adequate data fitting and reflects high prediction ability for the model's new estimation [37]. Moreover, the p-values for lack of fit ensure that the model is representing the experimental results at 95 % confidence limit, and a non-significant lack of fit is considered to be a good result because it reveals the model's pure error [37]. Additionally, Table 1 shows low values for the coefficient of variation (C.V. %), indicating precise and reliable experimental values. Residual plots are used in analyzing the model fit, estimating the problems of skewed distribution, outliers, and any non-random error present. In normal plots for the three responses, the residuals were found to be normally distributed to produce a straight line with the absence of outliers as illustrated in **(**Fig. S2**)**, supporting the model data accurately.

3.4. Experimental factors' effects

3.4.1. Effect of pH

In the initial experiments, it was discovered that there were no peaks were observed at pH 7.50, and earlier ZDA peak was observed at slightly acidic pH (4.5) that interfered with plasma peak at $\mathbf{R_t}$ 3.24 **(**Fig. S3**)**. Thus, prompting an investigation into the influence of mobile phase pH on chromatography. Three acidic pH levels, 2.5, 2.8, and 3, were examined to delve further into this. As a result, peaks were detected at those pHs. This suggests that LTZ (Pka 1.88), and ZDA (Pka 0.66) are more likely to be protonated at acidic pH values. In contrast, protonation is significantly reduced at the nearly neutral pH of 6.50, impacting detectability.

The use of the ***Box-Behnken Design*** revealed the impact of mobile phase pH on total retention time, RS1 and RS2. The total retention time was 8.598 min at the ideal pH of 2.8, with RS1 and RS2 14.8 and 5.4 respectively.

3.4.2. Effect of mobile phase composition

The study involved altering the mobile phase composition from 45 % acetonitrile and 55 % acidified water (v/v) to 55 % acetonitrile and 45 % acidified water. When the water content of the mobile phase was increased, the separation of ZDA and LTZ took a longer time, indicating that the mobile phase became more polar. The experimental design approach revealed that the composition of acetonitrile and water in the mobile phase had a significant impact on both total retention time and resolution, as shown in Table S2.

Table 1
Models' fitting statistical results.

Model term	Linear model		Quadratic model
	RS1 (Respone-1)	RS2 (Respone-2)	Run time (Respone-3)
R2	0.8588	0.6850	0.9903
Adjusted R2	0.8203	0.5991	0.9728
Predicted R2	0.7825	0.3589	0.9536
C.V.%	1.64	4.73	1.96
p-value (Lack of Fit)	0.4974	0.1755	0.5589

3.4.3. Effect of flow rate

The shortest total retention times were observed with the faster flow rate. It was found that the flow rate had a greater impact on run time but did not significantly affect the resolution between the drugs, as shown in Table S2.

3.4.4. Effect of two-factor interactions

The impact of two-factor interactions on CQAs was graphically interpreted through using response surface methodology like 2D-contour plots. The correlation between % acetonitrile with pH, flow rate, and % acetonitrile has a considerable impact on the resolution between ZDA and LTZ (RS1), as well as LTZ and TDF (RS2). An increase in resolution was observed at higher % acetonitrile (50–55 %) and lower flow rate (0.9) with almost no effect of pH (Fig. 2). The interaction between flow rate and % acetonitrile showed an effect on run time. The run time reduced as the flow rate and acetonitrile percentage dropped, with pH having little effect (Fig. 3).

3.5. Desirability function

The ultimate goal for optimization is to obtain the optimal condition giving the best responses values. Each separate response has its particular single desirability (d) that defines its specified objective from being minimize, maximize, or target the response. This was calculated separately for each response through a scale weighing from 0.10 to 10.0; high values indicate more significant responses than low values [37]. Afterward, combined desirability function (D) was achieved for all responses together, which ranges from D=0 defining an unacceptable response value to D=1 indicating a completely desirable value. Combined desirability function ensures an accurate, simple, and speedy choice regarding optimization, especially when multiple responses are existing [38]. The optimal chromatographic condition achieved was using a mobile phase of 0.1 % aqueous trifluroacetic acid (pH 2.8): acetonitrile (54.5:45.5, v/v) at 1.0 mL/min flow rate and at ambient temperature. It produces best resolution between ZDA, LTZ and TDF, as well symmetric peaks, with acceptable analysis run time.

3.6. System suitability testing parameters

After optimizing the chromatographic conditions and selecting the most effective parameters through using BBD approach; system suitability parameters were tested prior the validation process; ensuring the reproducibility and selectivity of the proposed method. Different parameters including column efficiency (**N**), resolution factor ($\mathbf{R_s}$), tailing factor ($\mathbf{T_f}$), capacity factor ($\mathbf{K'}$), and retention time ($\mathbf{R_t}$) were all calculated and found to be within the acceptable ranges [39] as shown in Table 2.

3.7. Method validation

Validation was performed according to the International Conference on Harmonization (ICH) Q2 guidelines [35].

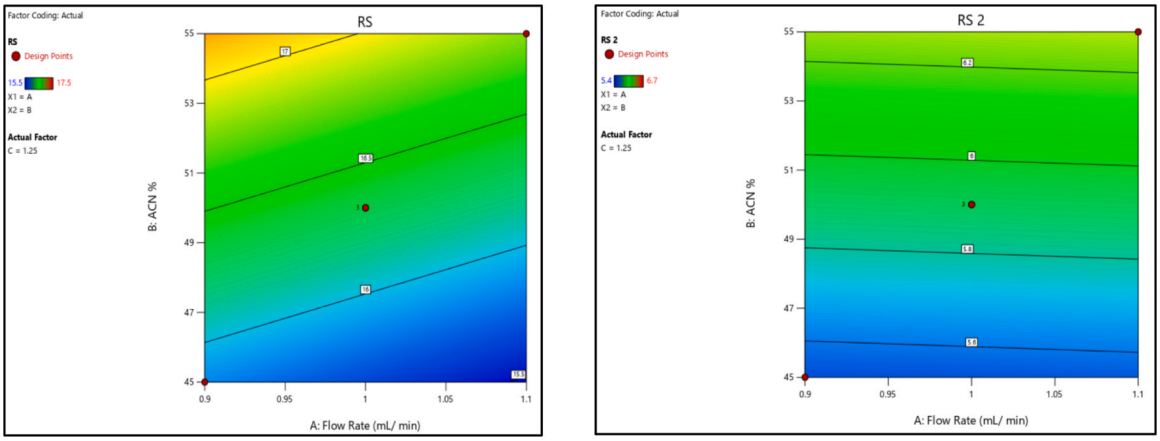
Under the optimum chromatographic conditions, linearity range was found to be 0.20–10.00 μg/mL for LTZ and ZDA (Figs. S4 and S5), obtaining acceptable correlation coefficient ($\mathbf{r^2}$) values; 0.9992 and 0.9999, approaching unity for both drugs respectively as represented in Table 3. LOD were 0.058 and 0.040 μg/mL while LOQ were 0.175 and 0.122 μg/mL for LTZ and ZDA respectively (Table 3). Accuracy of the proposed method was found to be 98.85 % for LTZ and 99.60 % for ZDA; existing within the acceptable range as shown in Table 3. Additionally, intra-day precision, inter-day precision and robustness were assessed separately through their adequately calculated RSD% values, indicating a precise and robust method (Table 3). Finally, selectivity was evaluated through good peaks' separation among the drugs in their laboratory-prepared mixtures. Besides, the chromatograms of LTZ and ZDA in their pharmaceutical dosage form (Femara® tablets and Zometa® vials) were similar to that produced by their corresponding intact drugs, and no additional peaks were observed (Table 4).

The standard solutions were stable when stored for 3 days at ambient temperature (32 ± 1 °C) and under refrigeration (8 ± 0.5 °C) in the optimized mobile phase. The solutions remained unchanged with no sign of degradation, based on peak areas' comparison with those obtained from freshly prepared solutions. Also, column variability was checked using the regular performance of system suitability testing for the prepared standard solutions, as well the column performance was found to be efficient and within the acceptable limits.

3.8. Application of the proposed method

The proposed method was successfully applied for the determination of the cited drugs in pharmaceutical dosage forms and spiked human plasma. According to the reported literature, TDF [18] and CBZ [9] were each assessed separately with the studied drugs, serving as internal standard (to minimize any elevated deviations and improve the precision results). As TDF is structurally related with LTZ, it showed better $\mathbf{R_s}$ with peaks' separation than CBZ, additionally, it gives apparently LTZ similar peak shape, response, and nearby retention time. Thus, it was the superior choice as an internal standard in this study.

The proposed method was applied to each pharmaceutical dosage forms (Femara® tablets and Zometa® vials), and the claimed results



(a) **(b)**

Fig. 2. Contour plots of (2a) RS1 and (2b) RS2 in terms of flow rate and ACN%.

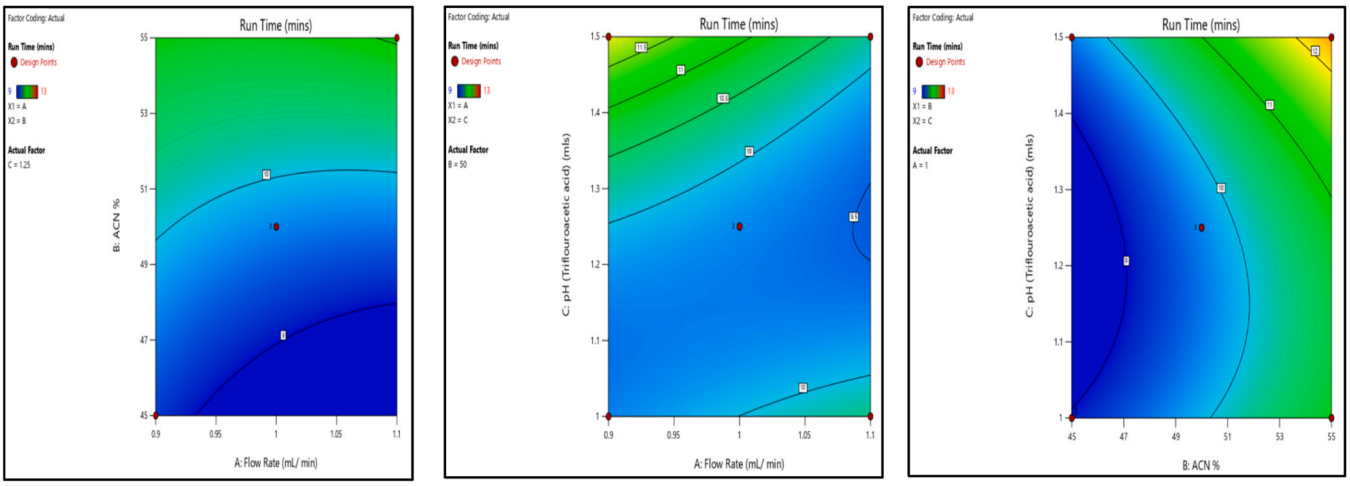


Fig. 3. Contour plots of Run Time in terms of (3a) flow rate and ACN%, (3b) flow rate and pH, and (3c) ACN% and pH.

Table 2
System suitability testing for the proposed HPLC method.

Parameters	ZDA	LTZ	TDF	Reference Value
Column efficiency "Number of theoretical plates" (N)	11,557.89	17,569.90	17,916.29	High value indicates more column efficiency
Resolution factor (Rs)	14.84		5.39	> 2
Tailing factor (T_f)	1.45	1.33	1.25	≤ 2
Capacity factor (K')	1.87	3.84	4.73	1–10
Retention time (Rt)	4.33	7.27	8.60	-

Table 3
Analytical validation data of the proposed method according to ICH guidelines.

Parameters	LTZ		ZDA	
Maximum wavelength (λ_{max}) (nm)	254		210	
Linearity range (μg/mL)	0.2–10			
Regression parameters				
Slope	0.1718		0.0917	
Intercept	0.0111		0.0045	
SE	0.872		0.482	
Correlation coefficient (r)	0.9992		0.9999	
LOD (μg/mL)	0.058		0.040	
LOQ (μg/mL)	0.175		0.122	
Accuracy* (Recovery%)	98.85		99.60	
Precision**	**Conc. (μg/mL)**	**RSD %**	**Conc. (μg/mL)**	**RSD %**
Inter-day	1.50	0.122	1.50	0.036
	3.00	0.142	3.00	0.021
	9.00	0.132	9.00	0.051
Intra-day	1.50	0.430	1.50	0.381
	3.00	0.377	3.00	0.288
	9.00	0.253	9.00	0.392
Robustness (RSD%)				
Trifluroacetic acid amount (± 0.025 %)	0.327		0.903	
% Organic phase (± 5 %)	1.238		1.557	

* n = 5.
** Relative standard deviation of three triplicate concentrations.

yielded 97.84 % ± 0.33 for LTZ and 103.73 % ± 0.94 for ZDA. This ensures method selectivity for both drugs without any interference from the excipients or other ingredients available (Table 4).

Concerning LTZ pharmacokinetics, it is orally bioavailable, absorbed from gastrointestinal tract, distributed by 55 %-60 % protein binding, slowly metabolized in liver as carbinol, and excreted through urine as glucuronide. LTZ reaches its maximum plasma concentration (C_{max}) at 41.22 ± 9.51 ng/mL [15]. Whereas, for ZDA, it is poorly bioavailable (administrated intravenous), rapidly absorbed by bones, distributed by 23–53 % protein binding, remained not metabolized in vivo, and excreted as intact through urine. ZDA reaches its maximum plasma concentration (C_{max}) at 471 ± 76.1 ng/mL [40].

The proposed method was applied for the determination of both drugs in spiked human plasma (Fig. 4). Protein-precipitation extraction technique was used for its simplicity, economical cost and easiness [8]. Acetonitrile was used as the precipitating solvent due to its reported efficient precipitation and fewer matrix effects [8], resulting in good recovery average percentages within acceptable ranges; 99.08 ± 2.25 for LTZ and 100.24 ± 1.43 for ZDA (Table 5). Plasma chromatograms confirm that the proposed method can be successfully applied on clinical experiments and real plasma samples (after being spiked with standard drug for LTZ determination).

The obtained results were compared statistically with the official and reported methods of the two studied drugs [2,28] using *student's t-test* and variance ratio *F-test* at 95 % confidence level. The calculated *t-values* and *F-values* are lower than the theoretical values showing absence of any significant difference considering the accuracy and precision (Table 6).

3.9. Method greenness assessment

Nowadays, it is very challenging and critical to compromise between increasing the analysis quality with respect to the corresponding environmental measures. Thus, greenness of the developed RP-HPLC method was evaluated using two different methods; GAPI [41] and AGREE [42].

Green Analytical Procedure Index (GAPI) is a semi-quantitative greenness tool, which evaluates and quantifies the ecological impact throughout 15 different aspects represented in a pictogram (contains of five pentagrams), classifying each stage in the analytical procedure from sampling process to final determination process. As well, it provides some qualitative data. GAPI's assessment takes place using a three colored-level scale (green, yellow, and red), indicating their high, medium, and low effects on the environmental impact respectively [41].

Analytical GREEness (AGREE) is a software based with greenness calculator, evaluating the 12 parameters mentioned by GAC [32]. This is

Table 4
Application of standard addition technique for the determination of LTZ and ZDA by the proposed method.

Pharmaceutical preparation	Femara® tablets Mean % ± SD 97.84 ± 0.33			Zometa® injection Mean % ± SD 103.73 ± 0.94		
	LTZ			ZDA		
	Claimed taken (µg/spot)	Added (µg/spot)	*Recovery % of added ± SD	Claimed taken (µg/spot)	Added (µg/spot)	*Recovery % of added ± SD
	2.00	2.00	98.01 ± 0.49	2.00	2.00	100.13 ± 1.27
	2.00	4.00	97.55 ± 0.99	2.00	4.00	99.98 ± 0.71
	2.00	6.00	100.63 ± 1.59	2.00	6.00	99.29 ± 1.01

* Average of three determinations.

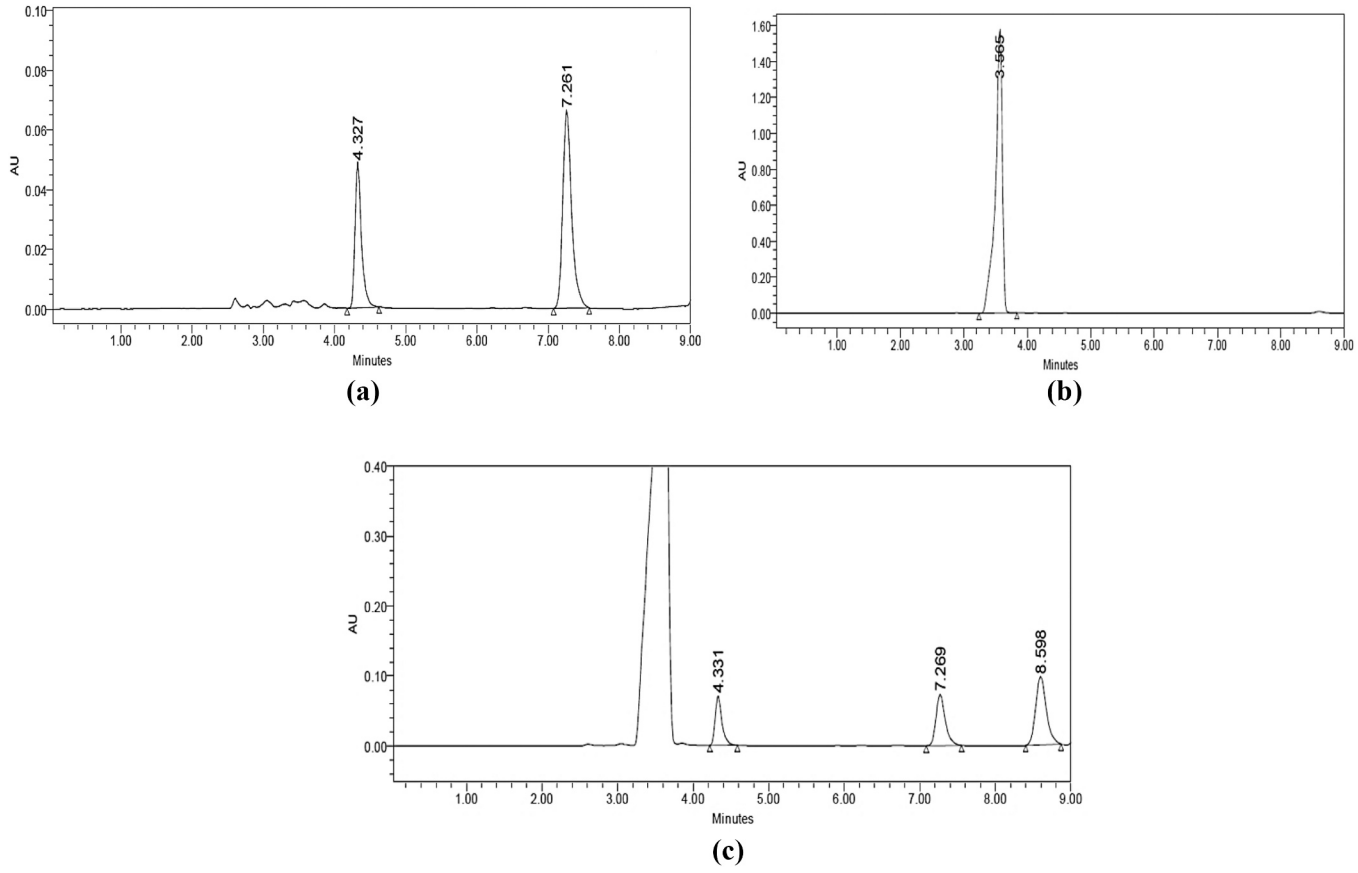


Fig. 4. Chromatograms of (4a) drug standards [R_t of ZDA = 4.327 and R_t of LTZ = 7.261], (4b) blank plasma [R_t = 3.565], and (4c) spiked plasma with drug standards [R_t of plasma = 3.565, R_t of ZDA= 4.331, R_t of LTZ= 7.269 and R_t of TDF = 8.598].

Table 5
Assay results for the determination of LTZ and ZDA in spiked human plasma by the proposed method.

Parameters	LTZ			ZDA		
	Amount taken (µg/mL)	*Amount found (µg/mL)	%Recovery	Amount taken (µg/mL)	*Amount found (µg/mL)	%Recovery
	0.20	0.20 ± 1.13	100.33	0.20	0.02 ± 0.86	102.19
	2.00	1.97 ± 0.21	98.59	2.00	0.19 ± 0.92	98.82
	6.00	5.77 ± 0.59	96.13	6.00	0.55 ± 0.74	99.69
	10.00	10.13 ± 1.12	101.25	10.00	0.92 ± 1.20	100.27

* Each result is the average of three determinations.

achieved by a weighing scale that ranges from 0.00 to 1.00, and the final greenness score is represented in a pictogram, besides, the performance of analytical procedure in every parameter with its weight given by the user can be assessed. Therefore, AGREE pictogram results are informative and easily interpreted [42].

A comparative greenness study was conducted between the proposed method and the reported methods [16,29] of each drug separately as represented in Table 7. It was found that the proposed method is much greener in terms of using simple extraction procedure i.e. no sample pretreatment or derivatizing agents were used. Also, for ensuring human

Table 6
Statistical analysis for the results obtained from the proposed method and the reference methods.

Statistical term	LTZ		ZDA	
	Proposed method	Reference method[a]	Proposed method	Reference method[b]
Mean%	98.85	98.40	99.60	98.15
SD[c]	2.47	1.77	1.36	1.41
SEM[d]	1.01	0.79	0.48	0.63
N	8	5	8	5
Variance	1.57	1.33	1.17	1.19
***Student's t-test* (2.201)**[e]	**0.338**		**1.845**	
***F-test* (6.09)**[e]	**1.94**		**1.07**	

[a] British pharmacopeial method.
[b] Reported chromatographic method.
[c] Standard Deviation.
[d] Standard Error of Mean.
[e] Values in parentheses are the *critical t-* and *F-values* at $P = 0.05$.

health safety, less hazardous solvents were chosen with very small quantities, causing solvent consumption. Moreover, using specifically HPLC instrumentation coupled with UV detector; guaranteeing its low KWt power needed in each run and thus, causing energy consumption. Finally, the waste produced from the proposed method was of low amounts; where they could be further treated either by degradation or passivation **(**Tables S3 and S4**)**.

3.10. Balance point display of the proposed method

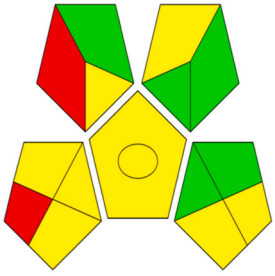
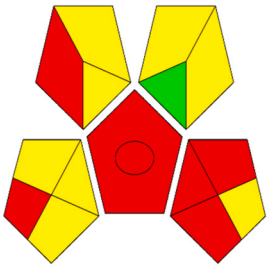
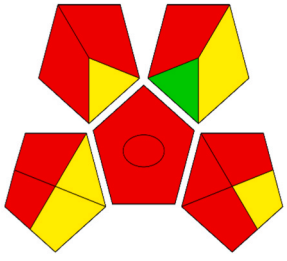
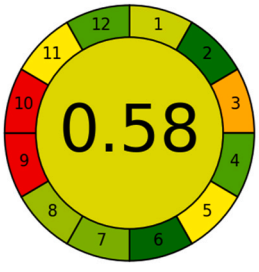
Our proposed chromatographic method will be assessed using the EVG tool to evaluate the balance between three important pillars, efficiency, validation, and greenness of the method. Each pillar receives a score ranging from 3 to zero for five evaluation criteria (A–E) [43]. The radar chart for the proposed EVG-HPLC method is shown in **(**Fig. 5**)**.

The first pillar of efficiency in experimental design includes the use of design of experiment (DOE) to reduce the number of experiments, while maximizing data yield and minimizing variability. In our method, the number of CMPs and CQAs were in equilibrium between higher values, which will increase design space and probabilities, and lower values which will keep the number of experiments doable. The second pillar of validation focuses on the type and magnitude of parameters used in method development and application, such as precision, LOQ, system suitability, and robustness. The third pillar of greenness in analytical chemistry emphasizes the use of multiple greenness tools which were two in our study GAPI and AGREE, also sample treatment procedures, reagents, solvents, instrumentation, energy consumption, and waste production. The grading for EVG pillars is shown in Table S5.

4. Conclusion

In this work, the integration between green chemistry and AQbD was employed; for developing a RP-HPLC method, regarding quantification of the studied co-administered drugs: Letrozole (hormonal anticancer drug) and Zoledronic Acid (antiresorptive drug). The novelty was beneficial since there were no any previously reported methods intended for this simultaneous determination. ***Box-Behnken Design*** was an effective choice for experimental optimization. Additionally, the method was validated in accordance with ICH requirements Q2, also it was found to be accurate, specific, and precise. This proposed method was successfully applied for the determination of the cited drugs in their pharmaceutical dosage forms (Femara® for LTZ and Zometa® for ZDA), as well, in spiked human plasma. Method greenness was assessed and compared with the reported literature of each drug separately, using two

Table 7
Comparative greenness assessment between the proposed method and the reference methods.

Greenness Method	Proposed method	LTZ reference method	ZDA reference method
GAPI			
AGREE			

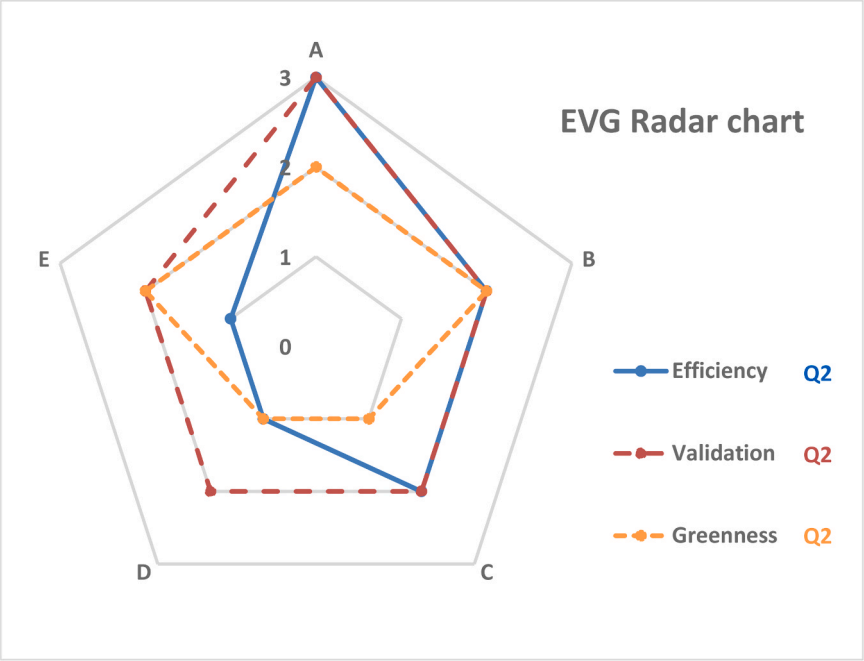


Fig. 5. EVG radar chart of the proposed method.

evaluation tools: Green Analytical Procedure Index (GAPI) and Analytical Greenness (AGREE). Also, a recent EVG tool was used to evaluate the balance between three important pillars, efficiency, validation, and greenness of the method.

Ethics approval and consent to participate

Ethics Committee approval from Cairo University number **AC 2754** was obtained. The study conducted in compliance with the International Guidelines for Research Ethics.

Competing Interest

The authors declare that there is no conflict of interest.

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CRediT authorship contribution statement

Nourhan A. Abd El-Fatah: Writing – original draft, Resources, Methodology. **Ghada M. El-Sayed:** Writing – review & editing, Supervision, Resources, Investigation, Formal analysis. **Manal Mohammed Fouad:** Writing – review & editing, Supervision, Investigation, Formal analysis. **Maha A. Hegazy:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. **Heba T. Elbalkiny:** Writing – review & editing, Resources, Methodology, Conceptualization.

Declaration of Competing Interest

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

Availability of data and material

The data are available from the corresponding author upon sensible request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpbao.2024.100036.

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