SPECTROFLUORIMETRIC METHOD FOR DETERMINATION OF LETROZOLE: ANALYTICAL APPLICATIONS TO BRAIN TISSUE SAMPLES AND ALKALINE DEGRADATION KINETIC STUDY

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A simple and sensitive spectroflourimetric method has been proposed for the determination of the antitumor agent letrozole in tablets, spiked human plasma, and rat brain tissue homogenates. Our method involves measuring the native fluorescence of letrozole at 590 nm upon excitation at 239 nm as indicated upon scanning its three-dimensional spectrum. Various experimental parameters were intensively studied and the method was validated as per ICH guidelines. The calibration curve was linear over the concentration range 5–160 ng/mL, with limit of detection 1.36 ng/mL. It was successfully applied to the analysis of letrozole in Femara® tablets with mean recovery 99.35 ± 1.49% and was further applied to study the alkaline degradation kinetics of letrozole. The pseudo first order rate constant and half-life were calculated. Moreover, Successful application of our proposed procedure was carried out on spiked human plasma and rat brain tissue samples. Linear ranges were found to be 5–30 and 10–130 ng/mL, with detection limits 1.25 and 1.71 ng/mL for plasma and brain samples, respectively. Thanks to the method's simplicity, selectivity, and high sensitivity, it can be used for routine analysis in quality control laboratories and for further clinical investigations involving letrozole.

Keywords: *letrozole, spectrofluorimetry, gliomas, rate constant, half-life.*

СПЕКТРОФЛУОРИМЕТРИЧЕСКИЙ МЕТОД ОПРЕДЕЛЕНИЯ ЛЕТРОЗОЛА: АНАЛИТИЧЕСКИЕ ПРИЛОЖЕНИЯ К ОБРАЗЦАМ ТКАНИ ГОЛОВНОГО МОЗГА И ИССЛЕДОВАНИЕ КИНЕТИКИ ЩЕЛОЧНОЙ ДЕГРАДАЦИИ

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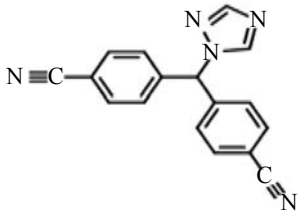
Предложен простой и чувствительный спектрофлуориметрический метод определения противоопухолевого вещества — летрозола — в таблетках, плазме крови человека и гомогенатах ткани головного мозга крыс. Метод включает в себя измерение собственной флуоресценции летрозола на длине волны 590 нм при возбуждении на 239 нм, что найдено в результате сканирования его трехмерного спектра. После исследования экспериментальных параметров метод валидирован в соответствии с рекомендациями Международного совета по согласованию технических требований к фармацевтическим препаратам для человека (ICH). Калибровочная кривая линейная в диапазоне концентраций 5–160 нг/мл с пределом обнаружения 1.36 нг/мл. Метод успешно применен для анализа летрозола в таблетках Фемара® со средним показателем восстановления 99.35 ± 1.49% и использован для изучения кинетики щелочной деградации летрозола. Процедура успешно применена к образцам плазмы крови человека и ткани головного мозга крысы. Установлены линейные диапазоны 5–30 и 10–130 нг/мл, пределы обнаружения 1.25 и 1.71 нг/мл для образцов плазмы и головного мозга.

Ключевые слова: *летрозол, спектрофлуориметрия, глиомы, константа скорости, период полураспада.*

Introduction. Letrozole, 4, 4′-(1H-1, 2, 4-triazol-1-yl methylene) bisbenzonitrile [1]



is a third generation nonsteroidal aromatase inhibitor. It was approved by the FDA in January 2001 for the treatment of estrogen-dependent breast cancer in postmenopausal women. After menopause, estrogen biosynthesis occurs mainly in peripheral tissues, such as breast, rather than in ovaries. The aromatase enzyme modulates the last step in estrogen biosynthesis through aromatization of androstenedione into estrogen. This increase in estrogen levels is widely known to be a high-risk factor for the development and growth of breast tumors. The mechanism of inhibition is attributed to the azole nitrogen in letrozole that bind reversibly to the heme iron of cytochrome P450 of the aromatase enzyme. In addition, a nitrile moiety in the para position acts as a bioisostere for the steroidal carbonyl group. Thus, letrozole mimics the backbone of the enzyme's natural substrate, androstenedione. That is why it shows high potency through a significant reduction in levels of circulating estrogens without affecting other steroidogenic pathways [2–4].

As a recent trend, letrozole has been a successful candidate in combating both primary and metastatic brain tumors from breast cancer. Case studies showed a dramatic reduction in tumor size along with prolonged patient survival upon daily dosing of letrozole either alone or combined with whole brain irradiation therapy [5–8]. Thus, letrozole has become a cornerstone in the field of oncology.

Various analytical methods have been reported for the analysis of letrozole in pure form, tablets, and biological fluids, either alone or in combination with its carbinol metabolites or other co-administered drugs as metformin, citalopram, fluoxetine, anastrazole, and tamoxifen. These methods include UV-spectrophotometry [9–12], RP-HPLC with UV detection [13–19] and fluorescence detection [20–22], LC-MS [23–25], GC-MS [26], capillary gas chromatography [27], capillary electrophoresis [12, 28–30], cyclic voltammetry [31], and potentiometric sensors [32]. Several chromatographic-stability-indicating methods showed that letrozole is highly sensitive towards alkaline degradation and stable towards acidic, oxidative, and photolytic conditions [33–35]. Our detailed literature review shows that only one spectrofluorimetric method has been reported for the analysis of letrozole in tablets [19]. Only HPLC methods with flourimetric detection have been proposed for determination of letrozole in biological fluids, also to the best of our knowledge, no analytical methods have been reported for the assay of the studied drug in brain tissue samples.

In this work, we have taken a different approach towards the analysis of letrozole. We aim to develop a validated simple, selective, and highly sensitive spectrofluorimetric determination of letrozole in tablets, human plasma, and rat brain tissue samples. This procedure was further used to follow up letrozole concentration upon studying its alkaline degradation kinetics.

Experimental. Spectroflourimetric analysis was carried out using Shimadzu spectrofluorimeter RF-6000 (Kyoto, Japan). Instrumental control and data acquisition were operated by LabSolutions software (Rev.B.04.01, Shimadzu).

Letrozole was purchased from China (Baoji Guokang Biotechnology Co. Ltd) with certified purity 99.50%. Femara® tablets, labelled to contain 2.5 mg letrozole per tablet, manufactured by Novartis Company, lot No. SH169 were purchased from the local market. Double distilled water and HPLC-grade methanol and acetonitrile (Sigma Gmbh, Germany) were used. Frozen human plasma was obtained from VACSERA (Giza, Egypt). Albino male (Wistar derived) Alderley Park strain rat brain tissue was used.

Stock solution (100 μg/mL) was prepared by dissolving the required amount of letrozole in methanol. Standard working solutions (1 and 5 μg/mL) were prepared by appropriate dilution from the previously mentioned stock (100 μg/mL) using methanol.

Procedure for calibration graph. Different aliquots of 1 μg/mL letrozole working solution were transferred into a set of 10-mL volumetric flasks and made up to the mark using acetonitrile:water mixture (30:70, %v/v) to obtain final concentrations in the range 5–160 ng/mL. Relative fluorescence intensity (RFI) for each solution was measured at 590 nm upon excitation at 239 nm. The calibration curve was constructed by plotting RFI versus final drug concentration, and the corresponding regression equation was computed.

Application to Femara® tablets. Ten Femara® tablets were weighed and finely powdered. An accurate weight equivalent to 10 mg letrozole was sonicated in 50-mL methanol for 10 min, filtered into 100-mL volumetric flask, and the residue washed several times using methanol, then made up to the mark with methanol to obtain a stock solution of concentration 100 μg/mL. Then, a working solution (1 μg/mL) was prepared as previously mentioned. An aliquot volume in the working concentration range was analyzed using the general analytical procedure, and standard addition technique was applied.

Application to spiked human plasma. In a set of 10-mL centrifuge tubes, different aliquots of 1 μg/mL letrozole working solution were spiked into 250 μL human plasma and vortexed for 60 s. Liquid-liquid extraction technique was applied using 3-ml diethyl ether. The mixture was vortexed for 2 min, followed by centrifugation at 6000 rpm for 20 min. Then 2-mL of the supernatant was evaporated to dryness, and the residue was reconstituted in 3 mL acetonitrile:water mixture (30:70, %v/v) to yield final drug concentrations in the range 5–30 ng/mL. RFI was measured at 588.6 nm upon excitation at 239 nm. A blank plasma experiment was done simultaneously. The calibration curve was constructed by plotting the RFI versus final drug concentration, and the corresponding regression equation was computed.

Application to spiked rat brain tissue homogenate. Rats were killed by cervical dislocation, and the brain was extracted from each and homogenized in saline (4 mL/gm) and frozen until use. In a set of 10-mL centrifuge tubes, different aliquots of 5 μg/mL letrozole working solution were spiked in 500 μL brain tissue homogenate and vortexed for 60 s. Liquid-liquid extraction technique was applied using 5 mL diethyl ether. The mixture was vortexed for 2 min, followed by centrifugation at 6000 rpm for 20 min. Then 4 mL of the supernatant was evaporated to dryness, and the residue was reconstituted in 5 mL acetonitrile:water mixture (30:70, %v/v) to yield final drug concentrations in the range 10–130 ng/mL. RFI was measured at 590 nm upon excitation at 239 nm. A blank brain tissue experiment was done simultaneously. The calibration curve was constructed by plotting the RFI versus final drug concentration, and the corresponding regression equation was computed.

Application to alkaline degradation kinetics. In a set of screw capped tubes, 1 mL of 5 μg/mL letrozole working solution was mixed with 4 mL 0.1 N NaOH. These mixtures were heated in a thermostatted water bath set at 80°C for different time intervals (10–60 min). At the specified time intervals, the contents of each tube were cooled and neutralized, and 1.6 mL of each was separately transferred into 10-mL volumetric flask. Then, the general analytical procedure previously mentioned was carried out and the concentration of each solution was derived from the previously computed regression equation.

Results and discussion. Letrozole is a native fluorescent compound. Upon scanning its 3D spectrum, maximum RFI was found to be at 590 nm upon excitation at 239 nm. Both excitation and emission wavelengths were chosen with respect to high sensitivity, maximum linearity, and best peak shape (Fig. 1). Our proposed method only differs from the published one [19] in the solvent used. A mixture of water:acetonitrile was used instead of methanol. However, experimental results reveal that our method outperforms the reported one [19] in terms of higher sensitivity, lower detection limit, and new analytical applications (Table 1).

Various solvents were tested including water, methanol, ethanol, acetonitrile, ethyl acetate, and DMSO. Also 0.1 N HCl, 0.1 N NaOH, and different buffer solutions, including acetate buffer (pH 3.5), borate buffer (pH 9.5), and phosphate buffer (pH 3.5, 7, 10), were tested. An obvious increase in RFI was seen upon using water, methanol, and acetonitrile. Thus mixtures of water:methanol and water:acetonitrile were tested. The water:acetonitrile mixture showed maximum fluorescence intensity, so it was tested in different ratios (Fig. 2).

TABLE 1. Comparison between Proposed Method and the Published One

Solvent	Proposed method	Method [19]
	Acetonitrile:water (30:70 % v/v)	Methanol
Sensitivity, ng/mL	5–160	40–240
LOD, ng/mL	1.36	9.617
Analysis in Femara® tablets	100.22± 0.64*	99.58 ±1.14*
Analysis in spiked human plasma	96.54 ± 9.33*	–
Analysis in spiked rat brain tissue	93.58 ± 9.37*	–

* Mean recovery% ± SD of five determinations of letrozole within concentration range.

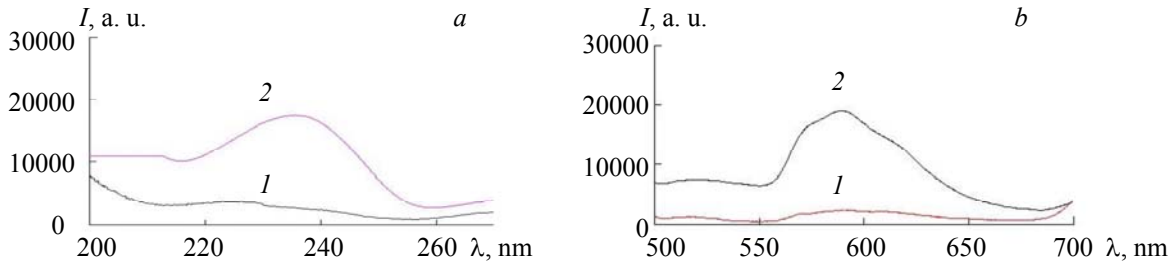


Fig. 1. Excitation (a) and emission (b) spectra of blank (1) and 160.00 ng/mL letrozole solution (2) showing peak at 239 (a) and 590 nm (b).

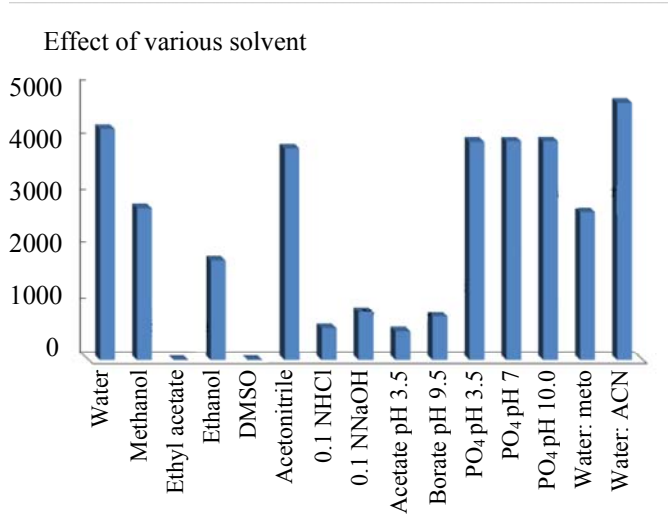


Fig. 2. RFI of 40.00 ng/mL letrozole using different diluting solvents at 590 nm upon excitation at 239 nm.

The acetonitrile:water mixture was tested in different ratios including 30:70, 50:50, and 70:30, %v/v. Acetonitrile:water, 30:70, %v/v, was the chosen diluting solvent with respect to sensitivity, reproducibility, best peak shape, and linearity over the required concentration range.

To enhance the sensitivity of the method, different organized media were tested, including nonionic surfactant (Triton-X 100), anionic surfactant (sodium dodecyl sulfate), cationic surfactant (cetrimide), and a macromolecule (β-cyclodextrin). They showed either negligible effect or even decreased the fluorescence intensity of letrozole. Thus, no organized medium was used in our study.

The proposed method was validated as per the ICH guidelines [36]. The validation parameters include linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, and specificity.

The calibration curve was found to be linear over the concentration range 5–160 ng/mL with correlation coefficient (r) 0.9994, indicating good linearity of the proposed method. The regression parameters are summarized in Table 2.

LOD and LOQ were calculated according to ICH guidelines using the equations LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$, where σ is the standard deviation of the intercept and S is the slope of the calibration curve. LOD and LOQ results are shown in Table 2.

The accuracy of the proposed method was assayed by analyzing pure samples of letrozole at five different concentration levels, and results were expressed as mean recovery% ± SD.

The precision was assayed by analyzing pure samples of letrozole at three different concentration levels on the same day for repeatability (intraday precision) and on three consecutive days for intermediate precision (interday precision). The results were expressed as %RSD, showing values less than 2%. This indicates the high precision of the proposed method (Table 2).

The specificity of the proposed method was assessed by analyzing letrozole in its pharmaceutical formulation. Excellent mean recovery% was obtained, indicating lack of interference from common tablet excipients (Table 2). The specificity was also proven by the absence of any interference from plasma and brain matrices or any other endogenous components at the selected wavelengths.

TABLE 2. Assay Validation Parameters of the Proposed Method

Parameter	Value
Range, ng/mL	5–160
Correlation coefficient, *r*	0.9994
Slope	96.17
Intercept	433.93
LOD, ng/mL	1.36
LOQ, ng/mL	4.12
Accuracy (mean recovery% ± SD)	99.60 ± 0.96
Repeatability (% RSD)	0.74
Intermediate precision (%RSD)	1.47
Specificity (mean recovery ± SD)	99.35 ± 1.49

The proposed method was applied for the analysis of letrozole in Femara® tablets. The validity of the method was also assessed by applying the standard addition technique. The results are shown in Table 3. The proposed method was statistically compared to the published method [19] using *F*-test and Student's *T*-test. The results of the standard addition technique were compared in both. The calculated *T* and *F* values were lower than theoretical ones, showing no significant difference between both methods (Table 4).

TABLE 3. Determination of Letrozole in Femara® Tablets 2.5mg and Application of Standard Addition Technique

Claimed conc	Found conc	Recovery % ± SD	Standard addition technique		
			Added, ng/mL	Found, ng/mL	Recovery%
Letrozole (40 ng/mL)	39.74 ng/mL	99.35 ± 1.49	10	10.01	100.10
			40	40.37	100.92
			70	69.75	99.64
Mean ± SD					100.22 ± 0.64

TABLE 4. Statistical Comparison of the Proposed Method

Parameter	Proposed method	Method [19]
Mean, %	100.22	99.58
SD	0.64	1.14
Variance	0.42	1.31
N	3	3
Students *t*-test (3.18)	0.83	–
F-test (19)	3.12	–

* The figures in parenthesis are the corresponding theoretical values for *F* and *t* at $p = 0.05$.

Application to spiked human plasma and rat brain tissue homogenate. Pharmacokinetic studies of letrozole revealed that the maximum plasma concentration, reached 1 h after a single 2.5 mg oral dose, is approximately 32 ng/mL, and the steady state concentration, reached after 2–6 weeks, is approximately 114 ng/mL [37].

Thanks to the high sensitivity achieved in our developed method, it was applied for the determination of letrozole in human plasma and rat brain tissue homogenate. Spiked samples were treated using a simple LLE method, and calibration curves were plotted. Validation parameters are summarized in Table 5.

Application to alkaline degradation kinetics. Previous stability studies showed that letrozole is highly sensitive towards alkaline degradation and resistant towards acidic, oxidative, and photolytic conditions. Partial hydrolysis of the cyano into the amide group occurs. Then complete hydrolysis into the carboxylic acid group occurs upon using high concentrations of NaOH. Annapurna et al. declared that upon heating letrozole with 0.1 M NaOH for 30 min at 80°C, only 42% of the drug was recovered [34]. In our present work, alkaline degradation kinetics was further studied by heating letrozole standard solutions with 0.1 M NaOH at 80°C for different time intervals. The degradation was found to follow pseudo first order since the stress

agent is always found in excess (Fig. 3). Rate constant (K) and halflife ($t_{1/2}$) were calculated from the equations:

$$S = -K/2.303, \quad (1)$$

$$t_{1/2} = 0.693/K, \quad (2)$$

where S is the slope of the curve representing the linear relationship between log$C$ (C is concentration) and time [38]. Results showed $K = 0.0479$ min^{-1} and $t_{1/2} = 14.46$ min.

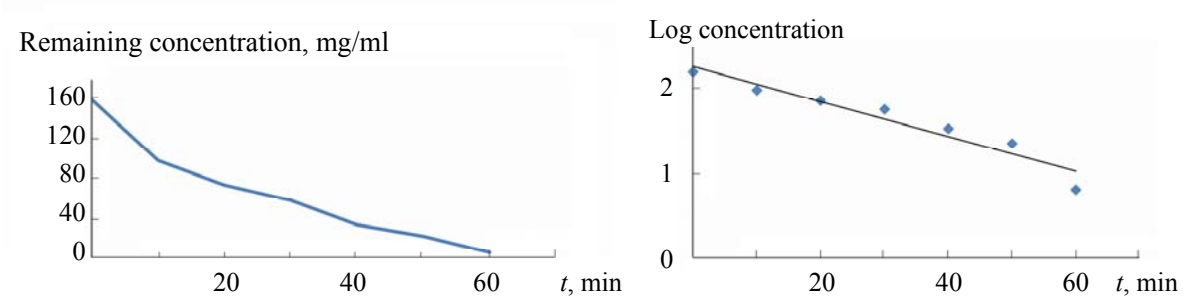


Fig. 3. A plot of remaining drug concentration at different time intervals.

TABLE 5. Validation Parameters of the Proposed Method in Spiked Human Plasma and Rat Brain Tissue Homogenate

Parameter	Human plasma	Rat brain tissue homogenate
Range, ng/mL	5–30	10–130
Correlation coefficient, r	0.9970	0.9992
Slope	31.20	348.24
Intercept	214.25	61.51
Accuracy (mean ± SD)	96.54 ± 9.33	93.85 ± 9.37
Repeatability (%RSD)	4.16	4.80
Intermediate precision (%RSD)	10.01	7.65
LOD, ng/mL	1.25	1.71
LOQ, ng/mL	3.81	5.19

Conclusion. A simple, sensitive, precise, and accurate spectrofluorimetric method has been developed and validated as per ICH guidelines. The goodness of this method allows its application in quality control laboratories for routine analysis of letrozole. Its high sensitivity in the nanoconcentration range allowed its application in spiked human plasma and spiked rat brain tissue homogenate. Thus, it is the first proposed spectroflourimetric method for analysis of letrozole in biological samples and the first analytical method proposed for its analysis in rat brain tissue homogenate. This method can be easily and readily applied to further clinical studies concerning letrozole. In addition, alkaline degradation kinetics was studied for the first time, and pseudo-first-order apparent rate constant and half-life were calculated.

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