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Research Article

DEPRESSION CONTROL IN BREAST CANCER PATIENTS: A NEW HPLC-UV METHOD FOR THE SIMULTANEOUS DETERMINATION OF THE CO-ADMINISTERED DRUGS, LETROZOLE AND VILAZODONE, IN HUMAN PLASMA

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ABSTRACT

Upon cancer diagnosis, patients frequently suffer a painful emotional reaction that progresses to a clinical depression. Adequate depression control increases patients' compliance to therapy and improves quality of life. This work proposes a new simple sensitive high performance liquid chromatographic method with UV detection for the simultaneous determination of the frequently co-administered anti-tumor agent; Letrozole and anti-depressant; Vilazodone in Human plasma. The proposed method employs a simple liquid-liquid extraction technique. The separation was carried out in a Phenomenex® RP-C_{18} column (250 x 4.6 mm, 5 um) and the mobile phase was potassium phosphate buffer (pH= 5.5) and methanol (50:50, v/v) with gradient flow rate conditions. UV detection was carried out at 240 nm for both drugs. Calibration curves were linear over concentration ranges 70-1000 and 140-1120 ng/ml for Letrozole and Vilazodone; respectively. Validation parameters such as linearity, accuracy, precision and stability were performed in accordance to FDA Bioanalytical validation guidelines. The proposed method is sensitive to determine the maximum plasma concentration of both drugs with adequate accuracy and precision, thus a valuable tool for further pharmacokinetic and pharmacodynamic studies.

Keywords: Depression; Cancer; Letrozole and Vilazodone.

1. INTRODUCTION

Diagnosis of breast cancer is an extremely unpleasant experience that may disrupt family life. Fear of Death may increase sensitivity to psychiatric disorders such as depression. Studies have shown a significant relationship between depression and increased perception of pain, suicidal ideas and wish for hastened death and low compliance to treatment. Much attention has to be paid to improving depression and its appropriate treatment. This reduces suffering, improves quality of life and even affects survival. Hence anti-carcinogenic drugs are frequently co-administered with anti-depressant agents.[1]

Letrozole, 4, 4'-(1H-1, 2, 4-triazol-1-yl methylene) bisbenzonitrile (Figure 1), a third generation non-steroidal aromatase inhibitor, was approved by the FDA in January 2001 for the treatment of estrogen dependant breast cancer in postmenopausal women. It showed high potency and tolerability through significant

reduction in levels of circulating estrogens without affecting other steroidogenic pathways.[2] Moreover, 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 a prolonged patient survival upon daily dosing of letrozole either alone or combined with whole brain irradiation therapy.[3-6]

Vilazodone, 5-[4-[4-(5-cyano-3-indolyl)-butyl]-1-piperazinyl] - benzofuran-2-carboxamide hydrochloride (Figure 1), is a new antidepressant with a novel mechanism of action, approved by the FDA in January 2011 for the treatment of major depressive disorder (MDD). Vilazodone is a dual acting serotonin re-uptake inhibitor and serotonin receptor (5-HT1A) partial agonist. It augments the availability of serotonin at both pre- and post synaptic sites, thus producing an antidepressant effect.[7]

In clinical practice, drugs that increase serotonin availability such as Vilazodone should be of the first resort during depression control in cancer patients due to its rapid onset, high tolerability and minimal side effects and drug-drug interactions. (8)

To date, various analytical methods have been reported for the analysis of letrozole in pure form, pharmaceutical formulation and biological fluids, either alone or in combination with its carbinol metabolites or other co-administered drugs. These methods include UV-spectrophotometry[9-12], Spectrofluorimetry[13], RP-HPLC with UV detection[13-18] and fluorescence detection[19-21], LC-MS[22-24], GC-MS[25], Capillary gas chromatography[26], capillary electrophoresis[12,27-29], cyclic voltammetry[30] and potentiometric sensors[31].

Also, several methods have been published for analysis of Vilazodone in bulk, pharmaceutical dosage form and biological samples including UV-visible spectrophotometric methods[32-37], spectrofluorimetric methods[38] and high performance thin layer chromatographic methods (HPTLC)[39]. Also high performance liquid chromatographic methods have been reported either with UV[40-42], fluorescence[38] or mass detection[43-47].

Co-administration of several drugs may present difference in the plasma drug concentrations of each. Thus monitoring of treatment requires the presence of sensitive, rapid and reliable methods of drug analysis in biological samples. On this approach, our Literature survey reveals the presence of published methods for the analysis of Letrozole with the anti-depressants; Fluoxetine[27] and Citalopram[20,28]. However, the literature is devoid of any analytical methods for simultaneous analysis of Letrozole and Vilazodone binary mixture.

HPLC technique has gained outstanding popularity in the field of analytical chemistry. This great position was achieved from its convenient separation of a wide range of sample types, low cost relative to other techniques, exceptional resolving power and speed and nanomolar detection levels.

Based on the several advantages offered by Vilazodone, we propose for the first time a simple, sensitive, reliable and precise HPLC method with UV detection for the analysis of this mixture of co-administered drugs in human plasma.

2. METHODS

2.1. Apparatus

Shimadzu LC-20AT HPLC (Shimadzu Corporation, Kyoto, Japan) fitted with SIL-20A autosampler, an on-line degasser DGU-20A3and SPD-20A UV-Visible detector. All components were controllable through LC-Solutions data software.

- Phenomenex® C-18 column (250mm x 4.6mm, 5um).

2.2. CHEMICALS AND REAGENTS

Vilazodone HCl was kindly supplied by El-Hikma Pharmaceuticals Co., Cairo, Egypt and certified to contain 99.10%. Letrozole was purchased from China (Baoji Guokang Biotechnology Co.Ltd) with certified purity 99.50%.

-HPLC-grade methanol, water and Di-ethyl ether. (Sigma, Gmbh, Germany). Frozen human plasma was obtained from VACSERA (Giza, Egypt). Phosphate buffer was prepared by

mixing different proportions of 1M K_2HPO_4 and 1M KH_2PO4 solutions.

2.3. Standard Solutions

Stock solutions of letrozole and Vilazodone (100 ug/ml) were prepared separately by dissolving 0.01 gm of each using methanol as solvent. Further dilution was done by separately transferring 0.50 ml from each stock into 10 ml volumetric flasks and volume was completed with methanol to obtain working solutions of concentration 5.00 ug/ml.

2.4. Procedures

2.4.1. Preparation of Spiked Calibration Standards and Quality Control Samples

Different aliquots of letrozole and Vilazodone standard working solutions were spiked in human plasma and vortexed for 30 sec.

4.00 ml diethyl ether were added to each, vortexed for 2 min at 1400 rpm then centrifuged at 6000 rpm for 15 min. 3.00 ml of the organic layer was transferred into a Wassermann tube and evaporated to dryness using eppendorf concentrator at 30°C under vacuum then reconstituted in 250.00 ul methanol: water mixture (50:50, v/v) with vortex mixing for 1 min.

2.4.2. Chromatographic conditions

Mobile phase chosen was methanol: potassium phosphate buffer (pH= 5.50) (50:50, v/v). All analyses were performed under gradient flow rate conditions as shown in (Table 1), at room temperature. UV detector was adjusted at 240 nm for quantitative determination of both drugs. All solvents were filtered through 0.45 um membrane filter immediately before use. Injection volume was 200.00 ul. Phenomenex® RP- C18 (250 mm x 4.6 mm, 5 um) column was equilibrated with mobile phase.

2.5. Method Validation

2.5.1. Linearity

Spiked plasma samples with concentrations equivalent to 70, 85, 140,400,800 and 1000 ng/ml letrozole and 140, 156, 280, 560, 800, 1120 ng/ml Vilazodone were prepared in triplicates as mentioned under 2.4.1 and analyzed under the selected chromatographic conditions shown under 2.4.2. The calibration curve was obtained by plotting average peak area versus concentration and the corresponding regression equation was computed.

2.5.2. Accuracy and Precision

Quality control samples were prepared in replicates as mentioned under 2.4.1 at LLOQ (70 ng/ml let and 140 ng/ml Vil), low QC (280 ng/ml let and 400 ng/ml vil.), mid QC (560 ng/ml for let and 600 ng/ml vil) and high QC (950 ng/ml let and 900 ng/ml vil.) and analyzed using the previously mentioned chromatographic conditions, on the same day and over three successive days as independent runs. Spiked concentrations were calculated from the corresponding regression equation and recovery percentages were calculated. Also Relative Standard Deviation (%RSD) was used to express intra- and inter-day precision of the proposed analytical method.

2.5.3. Selectivity

Blank plasma samples were analyzed and the resulting chromatograms were inspected for the occurrence of co-eluting peaks at the known retention times of both letrozole and Vilazodone. The retention times were 4.60 and 9.20 min for letrozole and Vilazodone, respectively.

2.5.4. Sensitivity

Sensitivity of the proposed method can be defined by the LLOQ, which is the lowest concentration of the analyte that can be quantitatively determined with acceptable accuracy and precision, regarded as the lowest calibrator level of the calibration curve(48).

2.5.5. Stability

Freeze and thaw stability study was carried out after two freeze (24 hr at -18°C) and thaw (4 hrs at room temperature) cycles. Bench top stability study was carried out leaving the prepared samples at room temperature for 4 hours before extraction and analysis. Stability studies were carried out at LQC and HQC concentration levels.

3. RESULTS AND DISCUSSION

3.1. Optimization of Chromatographic Conditions

In an attempt to develop the best separation of both drugs with optimum retention times, various solvents were tested as mobile phase. Acetonitrile: potassium phosphate buffer mixture and methanol: Potassium phosphate buffer mixtures were tested in various proportions, various buffer pH values and various flow rates in isocratic and gradient modes. When acetonitrile: potassium phosphate buffer mixture was used in various proportions, both peaks appeared at nearly the same retention time.

Upon studying the effect of mobile phase pH, we performed the analysis on three different pH values (3.50, 5.50 and 7.00). At pH 3.50, peaks were highly overlapped showing very poor separation. At pH 7.00, Vilazodone peak was significantly tailed. Optimum separation was achieved using methanol: potassium phosphate buffer (pH= 5.50) in a ratio 50:50, v/v under flow rate gradient conditions shown in (Table 1). It was found that increasing flow rate to 2.00ml/min improved the Vilazodone peak symmetry. The retention times were found to be 4.60 and 9.10 min for letrozole and Vilazodone; respectively. Detection was carried out at 240 nm which is λmax for both drugs. Obtained chromatograms from blank human plasma and spiked samples are shown in (Figure 2).

3.2. Sample Extraction Optimization

Various sample extraction techniques were tested to obtain maximum recovery. Protein precipitation technique using methanol was tested. Complete protein precipitation was achieved when increasing volumes of methanol. However, this diluted the sample, thereby decreasing the sensitivity of the assay. The chosen method was Liquid-liquid extraction using diethyl-ether as extracting solvent. Evaporation of organic solvent followed by reconstitution in a small volume achieved the best sensitivity to reach the steady state plasma concentration of both letrozole and Vilazodone. By reconstitution in methanol: water mixture, we obtained better peak resolution than in mobile phase.

3.3. Method Validation

3.3.1. Linearity

Calibration standards were prepared as recommended by the FDA guidelines in the same biological matrix as the study samples and a calibration curve was constructed for each analyte using six calibrator levels covering the quantitation range including LLOQ (48). A linear correlation was obtained between average peak areas and the corresponding spiked drug concentrations in the range 70-1000 ng/ml for letrozole and 140-1120 ng/ml for Vilazodone. Peak area-concentration relationship was fit to the simplest regression model.

3.3.2. Accuracy and Precision

''Accuracy is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions, whereas *Precision is the closeness of agreement (i.e., degree of scatter) among a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions*(48). According to FDA guidelines, accuracy and precision were studied by preparing quality control samples on four concentration levels: LLOQ (lowest level of calibration curve), LQC (three times LLOQ), MQC (mid-range) and HQC (high-range). The obtained values were within ± 15% of nominal concentration and 20% at LLOQ, which proves the accuracy of the proposed method. Also Intra- and Inter- day precision results were within acceptable limit, which is ± 15% RSD and 20% at LLOQ.

Accuracy and precision results are shown in (Tables 2 and 3). Results of all validation parameters are shown in (Table 4).

3.3.3. Selectivity

Selectivity is the extent to which the method can determine a particular compound in the analyzed matrices without interference from matrix components. Blank human plasma samples showed no interfering peaks at the retention times of both drugs as shown in (Figure 2).

3.3.4. Stability

Bench-top stability should assess the stability of analyte under the expected laboratory handling conditions for study samples. Freeze/thaw stability should assess the stability of analyte after several freeze and thaw cycles. Stability studies were performed on two concentration levels (LQC and HQC) and results obtained from stability QCs are compared to those obtained from freshly prepared QCs.
Obtained concentration values, shown in (Table 5) meet the FDA acceptable limits which are ± 15% of the nominal concentration. This confirms the high stability of letrozole and Vilazodone throughout handling and storage.

3.4. System Suitability

System suitability parameters for the proposed chromatographic method were calculated and results, shown in (Table 6), shows that the instrumental system is performing properly.

3.5. Statistical Analysis of Results

The results obtained by the proposed method were statistically compared to published methods (23,46) using student's t-test and variance ratio F-test at P= 0.05. The calculated t- and F-values didn't exceed the theoretical values, which indicate that there is no significant difference between both methods in the determination of both Letrozole and Vilazodone in human plasma. (Tables 7 and 8)

4. CONCLUSION

The proposed RP-HPLC method is the first developed method for the analysis of Letrozole and Vilazodone mixture. It offers various advantages in terms of simplicity, sensitivity, accuracy and short analysis time. Thus it could be easily applied in routine analysis of this mixture in human plasma. It can also be applied for further clinical studies.

Table 1: Gradient Flow Rate Conditions of the Proposed HPLC-UV Method

Time	Flow rate
0-6.50	1.50 ml/min
6.51-10.50	2.00 ml/min
10.51-15.00	1.50 ml/min

Table 2: Accuracy and Precision Results of Letrozole Obtained by Analysis of Quality Control Samples using the Proposed HPLC-UV Method

		Intra-day			Inter-day		
	QC level (ng/ml)	Mean recovery %	SD	% RSD	Mean recovery %	SD	% RSD
LLOQ	70.00	105.05	2.94	2.79	100.87	7.53	7.46
LQC	280.00	99.61	1.50	1.50	96.64	5.24	5.42
MQC	560.00	95.29	5.80	6.08	89.42	10.96	12.25
HQC	950.00	104.63	2.64	2.52	97.89	11.82	12.07

SD: Standard deviation
%RSD: Relative Standard Deviation

Table 3: Accuracy and Precision Results of Vilazodone Obtained by Analysis of Quality Control Samples using the Proposed HPLC-UV Method

		Intra-day			Inter-day		
	QC level (ng/ml)	Mean recovery %	SD	% RSD	Mean recovery %	SD	% RSD
LLOQ	140.00	100.40	2.33	2.32	96.39	8.22	8.52
LQC	400.00	109.77	5.59	5.09	111.85	5.34	4.77
MQC	600.00	105.25	7.43	7.05	108.34	7.49	6.91
HQC	900.00	105.05	6.84	6.51	97.56	13.84	14.18

Table 4: Results of Assay Validation Parameters of the Proposed HPLC-UV Method for Determination of Letrozole and Vilazodone in Human Plasma

Parameter	Letrozole	Vilazodone
Linearity (ng/ml)	70.00-1000.00	140.00-1120.00
Slope	2651.0687	2796.9481
Intercept	-32372.0858	-168341.0636
Correlation coefficient (r)	0.9996	0.9998
LLOQ (ng/ml)	70.00	140.00
Standard error of intercept (Sa)	19502.83	14488.52

Table 5: Results of Stability of the Proposed HPLC-UV Method

	Letrozole				Vilazodone			
	Conc. (ng/ml)	Mean recovery %	SD	% RSD	Conc. (ng/ml)	Mean recovery %	SD	% RSD
Freeze/ thaw	280.00	89.19	12.31	13.80	400.00	111.85	14.12	12.62
	950.00	101.24	16.12	15.92	900.00	93.39	15.26	16.34
Bench top	280.00	91.53	11.09	12.11	400.00	114.83	9.60	8.36
	950.00	103.55	14.87	14.36	900.00	91.40	12.45	13.62

Table 6: System Suitability Parameters for the Analysis of Letrozole and Vilazodone using the Proposed HPLC-UV Method

Parameter	Value for letrozole	Value for Vilazodone	Reference value
Capacity factor (k')	1.77	4.58	≥ 2
Tailing factor (T)	1.43	2.05	≤2
Number of theoretical plates	3238.08	2613.59	(> 2000)
HETP	46.32	57.39	The smaller the value, the higher the column efficiency
Resolution	8.19	8.88	≥ 2
Selectivity factor	--	2.57	(>1)

Table 7: Statistical Comparison of the Results Obtained by Applying the Proposed Method and the Reported Method for the Analysis of Letrozole in Spiked Human Plasma

Parameter	Proposed method	Reported method[23]
Mean	100.44%	105.34%
SD	6.73	4.51
Variance	45.29	20.43
n	4	4
Students t-test (2.57)	1.20	--
F(9.27)	2.21	--

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

[23]Development and validation of a liquid chromatography– tandem mass spectrometry method for the simultaneous quantification of tamoxifen, anastrazole, and letrozole in human plasma and its application to a clinical study.

Table 8: Statistical Comparison of the Results Obtained by Applying the Proposed Method and the Reported Method for the Analysis of Vilazodone in Spiked Human Plasma

Parameter	Proposed method	Reported method[46]
Mean	105.11%	99.72%
SD	3.82	2.01
Variance	14.64	4.04
N	4	4
Students t-test (2.57)	2.49	--
F(9.27)	3.62	--

* The figures in parenthesis are the corresponding theoretical values for F and t at p= 0.05.
[46]UPLC–MS-MS Method for the Determination of Vilazodone in Human Plasma: Application to a Pharmacokinetic Study.

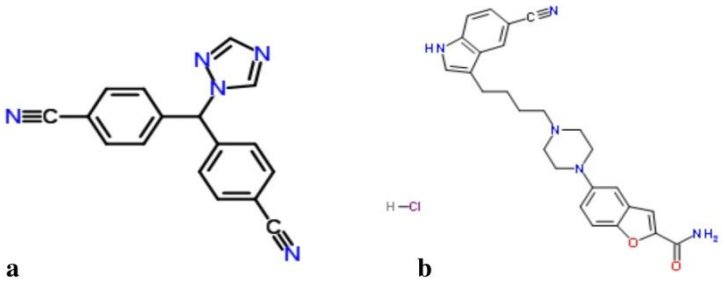

Fig. 1: Chemical structure of (a) Letrozole and (b) Vilazodone HCl

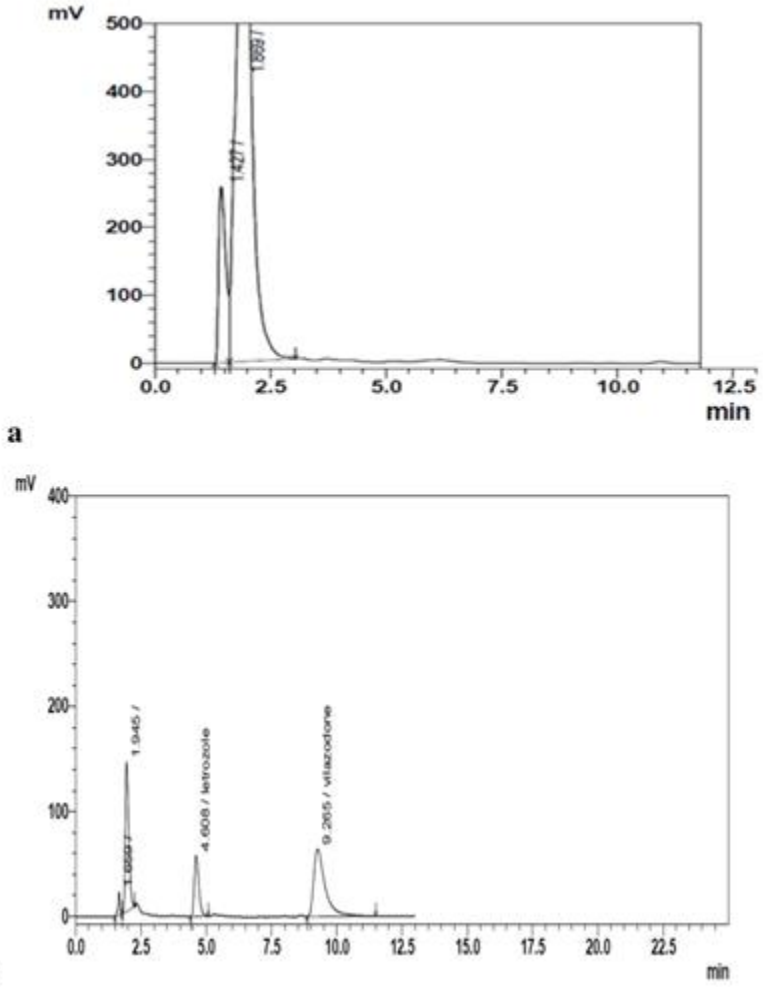

Fig. 2: Obtained Chromatograms of (a) blank human plasma and (b) separation of Letrozole and Vilazodone at 240nm

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