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RESEARCH ARTICLE

Analytical Method Validation for HPLC Assay of Oral Anticancer Drug Exemestane

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Summary

Exemestane (EXE) is an irreversible aromatase inactivator used for the treatment of postmenopausal women with advanced breast cancer. EXE is practically insoluble in water and its bioavailability is approximately 5%. It is known that cyclodextrin (CD) complexation can enhance water solubility and intestinal permeability and thus the oral bioavailability of poorly soluble drugs. Therefore, it was aimed to design and develop CD complexes containing EXE to improve the aqueous solubility and increase the intestinal permeation in order to enhance the oral bioavailability. In this study, an analytical method developed by Yu et al. was validated to determine the amount of soluble EXE in phase solubility studies and in vitro dissolution studies of EXE. An HPLC method with UV detection was validated in methanol medium, using a Hichrom Nucleosil 100 C18 column (150mmx4.6mm). EXE peak area was linear ($r^2 > 0.9996$) over the concentration range 2.5 – 50 µg/mL. This validation included specificity, range, linearity, precision, accuracy, LOD and LOQ; all results were acceptable and confirmed that the method is suitable for its intended use.

Key Words: *HPLC, validation, exemestane, dissolution, bioavailability, cyclodextrin, inclusion complex.*
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Oral Antikanser İlaç Ekzemestan'ın HPLC ile Tayini İçin Analitik Yöntem Validasyonu
Özet

Ekzemestan (EXE), menopoz sonrası ilerlemiş meme kanserinin tedavisinde kullanılan bir aromataz inaktivatörüdür. EXE pratik olarak suda çözünmez ve biyoyararlanımı %5 dolaylarındadır. Siklodekstrinlerle kompleksleşmenin suda çözünürlük ve intestinal permeabiliteyi ve dolayısıyla da çözünürlüğü düşük ilaçların biyoyararlanımını arttırdığı bilindiğinden EXE'ın sudaki çözünürlüğünü arttırmak ve intestinal permeasyonunu yükselterek oral biyoyararlanımını iyileştirmek amacıyla, EXE içeren siklodekstrin (CD) kompleksleri hazırlanması amaçlanmıştır. Bu çalışmada, faz çözünürlük çalışmaları ve dissolüsyon çalışmalarında, çözünmüş EXE'ın miktar tayininde kullanılmak üzere, C. Yu ve arkadaşları tarafından geliştirilmiş olan bir analitik yöntemin validasyonu yapılmıştır. EXE'ın metanoldeki çözeltisi için, Hichrom Nucleosil 100 C18 kolon (150mmx4,6mm) ve UV deteksiyon kullanılarak bir HPLC yöntemi valide edilmiştir. EXE pik alanları, 2.5 – 50 µg/mL konsantrasyon aralığında doğrusal bulunmuştur ($r^2 > 0.9996$). Bu validasyon ile özgünlük, aralık, doğrusallık, kesinlik, doğruluk, teşhis limiti ve tayin limiti saptanmış ve tüm sonuçlar kabul edilebilir ve kullanım amacına uygun bulunmuştur.

Anahtar Kelimeler: *HPLC, validasyon, Ekzemestan, çözünürlük, biyoyararlanım, siklodekstrin, inklüzyon kompleksi.*

INTRODUCTION

Breast carcinoma has become a major health problem over the past 50 years, affecting as many as one in eight women[1,2]. Although there have been substantial developments in the treatment of breast cancer, approximately 25% of women with breast carcinoma eventually will die from the disease [3].

It has long been recognized that approximately one-third of all breast carcinomas are estrogen-dependent and will regress after estrogen deprivation[4]. Thus, reducing the level of estrogen remains a valuable target for breast carcinoma treatment in both premenopausal and postmenopausal women. Reducing the effects of estrogen can be mediated by agents that block estrogen at the receptor level with inhibitors of estrogen biosynthesis, such as the aromatase inhibitors[5].

Exemestane (EXE) is a new hormonal agent recently approved by the Food and Drug Administration

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(FDA) for the treatment of breast cancer and is marketed as Aromasin®. EXE is an irreversible aromatase inactivator used for the treatment of postmenopausal women with advanced breast cancer[6]. This drug is orally active and a potent inhibitor of peripheral aromatase activity[7,8].

Aromatase is a complex enzyme consisting of two proteins: the aromatase cytochrome P450, a hemoprotein, and reduced nicotinamide adenine dinucleotide diphosphate (NADPH) cytochrome P450 reductase, that donates electrons to the P450 aromatase[9]. EXE is a white to slightly yellow crystalline powder with a molecular weight of 296.41. Chemical structure of EXE (Fig. 1) is 6-methylenandrosta-1,4-dien-3,17-dion[10]. EXE binds covalently to the active site cytochrome P450, making it inactive[11]. The recommended therapeutic dose for EXE is 25 mg per day[12,13].

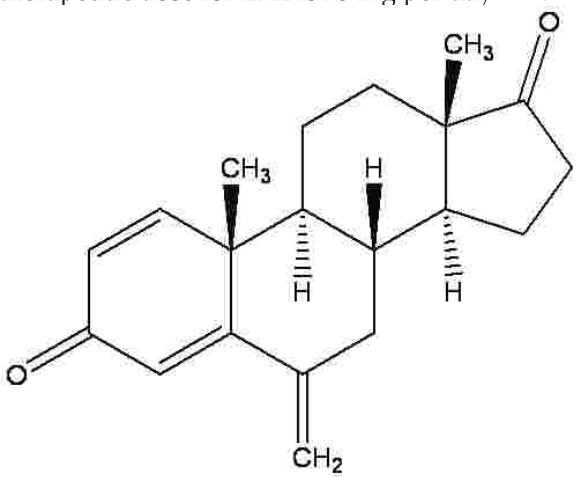

Figure 1: Chemical structure of EXE.

EXE is freely soluble in N,N-dimethylformamide, soluble in methanol, and practically insoluble in water[14]. EXE has been developed for oral administration. Treatment with EXE administered orally has been shown to be well tolerated by patients. Following oral administration of radiolabeled EXE, only 42% of radioactivity was absorbed from the gastrointestinal tract, due to its low solubility in water[13]. Preclinical data obtained in rats and dogs in which EXE was given i.v. indicated that the absolute bioavailability was about 5%[15].

In the past, three high performance liquid chromatographic (HPLC) methods for plasma determination of EXE were developed: one with UV detection (with limit of quantitation [LOQ] 10 ng/ml)[16], and the others with mass spectrometric detection (with LOQ 1 ng/ml[17] and 0.25 ng/ml[18]). The purpose of this study was to determine the analytical validation and feasibility of the modified HPLC method first developed and validated by Yu et al.[19] for EXE, which we can use in our in vitro studies. We aimed to determine the amount of soluble EXE using this HPLC method in phase solubility studies and to quantify the in vitro dissolution of EXE from cyclodextrin (CD) complexes using this analytical method.

MATERIALS and METHODS

Materials

HP Agilent 1100 series (Germany) was used for the validation of the method. EXE was supplied from Pfizer. HP-β-CD and HP-γ-CD were purchased as Cavasol® (Wacker Chemicals, USA) and β-CD was purchased as Kleptose® (Roquette, France). Sodium lauryl sulphate (SLS) was purchased from Merck (Germany). HPLC grade acetonitrile and HPLC grade methanol were purchased from Sigma Aldrich (Germany). Purified water was obtained from Water Lab System, Millipore (France). All other reagents were of HPLC grade and were used without purification.

Method

An HPLC method with UV detection that was developed by Yu et al.[19] was selected for the method of analysis. The reversed-phase procedure utilized a Hichrom Nucleosil 100 C18 column (150mmx4.6mm) and UV detection at 249 nm. The column temperature was maintained at 40°C. The mobile phase contained acetonitrile and purified water (44:56 v/v). The flow rate was 1.2 mL/min for 13 min of assay time with an injection volume of 10 μL.

Standard solution (250 μg/mL) of EXE was prepared in methanol. Methanol was selected as the solvent because of EXE's high solubility.

Detection wavelength 249 nm was selected because

it gives a UV maximum and provides the sensitivity needed for quantification of EXE (Fig. 2).

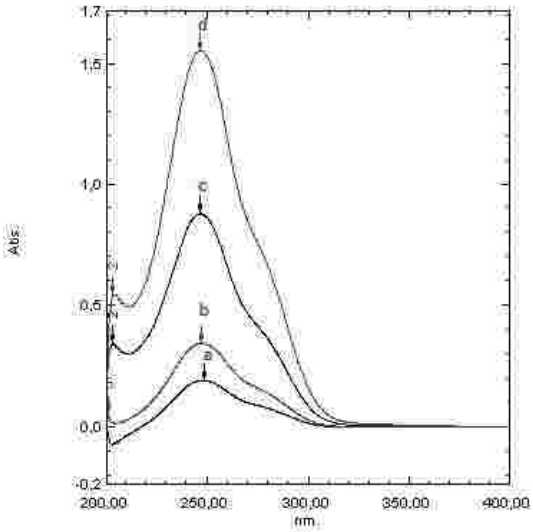

Figure 2: UV spectra of EXE in methanol solutions (a- 2.5 μg/mL, b- 6.25 μg/mL, c- 12.5 μg/mL, d- 25 μg/mL).

Specificity

Specificity was examined by analyzing dissolution medium and solutions of CDs, which were to be used to prepare EXE inclusion complexes. EXE's spectrum was compared against those of β-CD, HP-β-CD, HP-γ-CD, 0.5% SLS solution and pH 7.4 phosphate buffer.

Range

The range of an analytical procedure is the interval between the upper and lower concentrations of analyte in the sample, for which it was demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity[20].

Based on linearity, accuracy and precision data, the validated range of the method is from 10% to 200% of the maximum EXE concentration in dissolution medium (2.5 - 50 μg/mL EXE).

Linearity

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample[20]. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example by calculation of a regression line by the method of least squares[21].

Six concentrations between 2.5–50 μg/mL of EXE solutions were prepared to show the linearity of the method. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares were calculated.

Precision

There are three categories of precision—repeatability, reproducibility and intermediate precision. Repeatability is the precision of the method under the same operating conditions over a short period of time. Reproducibility determines the precision between laboratories. Intermediate precision is a measure of intra-laboratory variance using different operators on different days, equipment, etc., and is not required in cases where reproducibility has been performed[20].

Precision is usually expressed as the variance, standard deviation or coefficient of variation (CV) of a series of measurements[20]. Precision was evaluated by performing repeatability (instrument and method precision) and intermediate precision (variation between days).

Accuracy

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range. Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.[21].

Samples at 40, 80 and 120% of the nominal assay concentration (10, 20 and 30 μg/mL) were prepared for accuracy testing of 25 mg tablets. Six preparations were made for each concentration. Percent recoveries were calculated.

Limit of Detection / Limit of Quantification

Limit of detection (LOD) and limit of quantitation (LOQ) were measured based on the signal-to-noise ratio.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected and quantified[21].

RESULTS and DISCUSSION

Validation of HPLC Method

The method was validated to meet requirements for a global regulatory filing and this validation included specificity, range, linearity, precision, accuracy, LOD and LOQ.

Specificity

EXE's spectrum was compared against β-CD, HP-β-CD, HP-γ-CD, 0.5% SLS solution and pH 7.4 phosphate buffer. Absence of interference is demonstrated in Figures 3a - 3f.

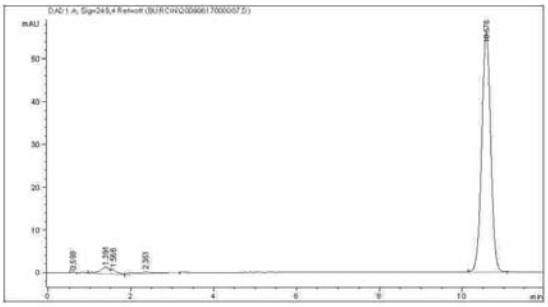

Figure 3a : Chromatogram of 20 μg/mL EXE.

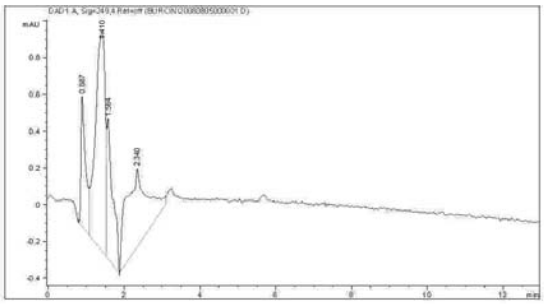

Figure 3b : Chromatogram of 200 μg/mL β-CD.

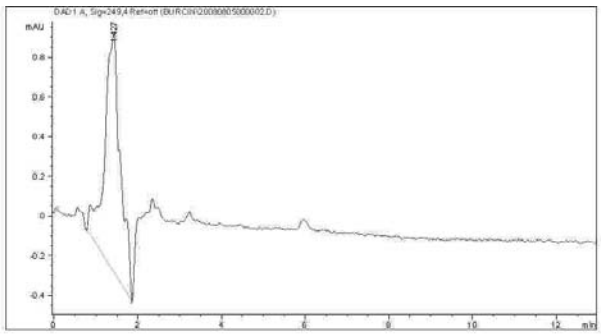

Figure 3c : Chromatogram of 200 μg/mL HP-β-CD.

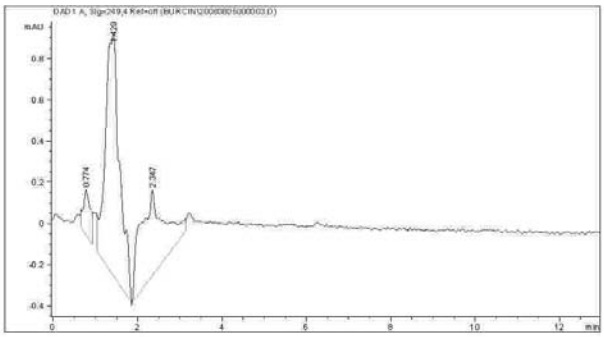

Figure 3d : Chromatogram of 200 μg/mL HP-γ-CD.

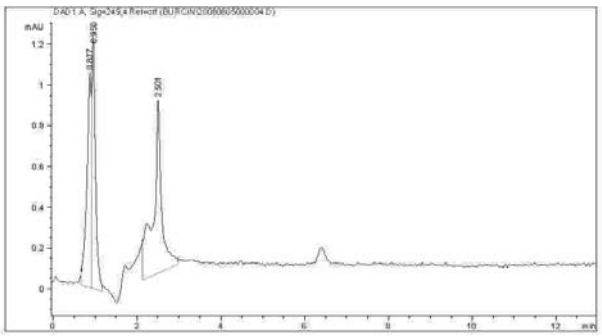

Figure 3e : Chromatogram of 0.5% SLS solution.

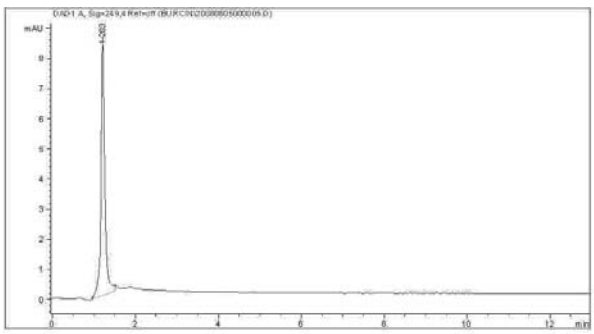

Figure 3f : Chromatogram of pH 7.4 phosphate buffer.

Range

A standard solution (250 μg/mL) of EXE was prepared in methanol and subsequently diluted with methanol

down to concentrations between 2.5 μg/mL and 50 μg/mL.

This range covers the in vitro working range for EXE.

Linearity

Data indicated that the EXE peak area is linear over the concentration range of 2.5–50 μg/mL. The r^2 for the regression line is 0.9996 with a slope of 28.498±2.27 and a y-intercept of +22.586±8.74. The y-intercept is within 1.55% of the response at the 100% level. These results were considered acceptable.

The regression line and equation are shown in Figure 4.

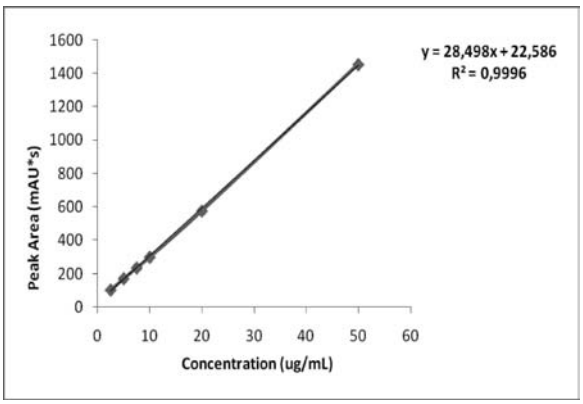

Figure 4 : The regression line for EXE (2.5–50 μg/mL).

Precision

Six samples were prepared at the same concentration (10 μg/mL) to evaluate method precision and CV% was 1.83%, which was considered acceptable.

Six injections of the same sample (10 μg/mL) were made to evaluate instrument precision. CV% was 0.725%, which was considered acceptable.

Intermediate precision was evaluated to show the variation between the days. Samples (10 μg/mL) were prepared on 3 separate days and CV% was 0.294%, which was considered acceptable.

Results are shown in Table 1.

Accuracy

Six preparations were made for each concentration. Recovery of EXE was determined for each sample. Results are shown in Table 2.

CVs were <2% for each concentration, which were considered acceptable. Average percent recoveries were calculated for each concentration and the results were 94.10% for 10 μg/mL, 95.62% for 20 μg/mL and 95.77 % for 30 μg/mL.

Limit of Detection / Limit of Quantification

LOD and LOQ were measured based on the signal-to-noise ratio. LOD was found as 21 ng/mL and LOQ as 71 ng/mL.

CONCLUSIONS

EXE has great potential as an oral anticancer agent for the treatment of breast cancer. It has been shown to be well tolerated in controlled clinical trials and

Table 1. Precision results

Samples (10 μg/mL)	Method Precision		Instrument Precision		Intermediate Precision	
	Measured Peak Areas (mAU*S)	Retention times	Measured Peak Areas (mAU*S)	Retention times	Measured Peak Areas (mAU*S)	Retention times
1	276.71	10.11	338.44	10.01	289.59	10.18
2	283.49	10.11	343.32	10.01	289.17	10.09
3	271.62	10.11	343.74	10.03	287.95	10.13
4	270.42	10.12	338.93	10.03	-	-
5	279.77	10.12	343.15	10.03	-	-
6	279.47	10.14	343.62	10.03	-	-
Mean	276.91	10.12	341.87	10.02	288.90	10.13
CV %	1.83	0.11	0.73	0.11	0.29	0.45

Table 2. Percent recoveries and coefficients of variation

Concentration/ Samples	10 μg/mL		20 μg/mL		30 μg/mL	
	RECOVERY%	BIAS%	RECOVERY%	BIAS%	RECOVERY%	BIAS%
1	93.86	6.14	94.76	5.24	96.82	3.18
2	95.60	4.40	95.28	4.72	95.43	4.57
3	94.27	5.73	95.83	4.17	94.01	5.99
4	95.28	4.72	97.91	2.09	97.23	2.77
5	94.10	5.90	95.62	4.38	95.77	4.23
6	94.11	5.89	95.66	4.34	93.36	6.64
Mean	94.53	5.47	95.85	4.15	95.44	4.56
CV %	0.76		1.13		1.59	

has a more favorable tolerability profile than standard second-line options for treatment of advanced breast cancer.

In this study, validation of an analytical method for EXE quantification was evaluated for specificity, range, linearity, precision, accuracy, LOD and LOQ in order to establish the suitability of the analytical method.

It is known that CD complexation can enhance intestinal permeability and thus the oral bioavailability of poorly soluble drugs[22,23]. It has been shown that the poorly-soluble antiestrogen drug tamoxifen used in the treatment of metastatic breast cancer can be complexed to both natural and amphiphilic β-CDs[24]. Thus, it was aimed to design and develop CD complexes containing EXE to improve their aqueous solubility and increase the intestinal permeation in order to enhance the oral bioavailability. A current study is under way for preparing EXE:CD inclusion complexes that can be useful for increasing the bioavailability of EXE. This HPLC method was found feasible for determining and quantifying EXE in phase solubility, dissolution and stability studies

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