Advanced Pharmaceutical Bulletin

Adv Pharm Bull, 2016, 6(2), 251-256
doi: 10.15171/apb.2016.034
http://apb.tbzmed.ac.ir

Research Article

A Simple, Fast, Low Cost, HPLC/UV Validated Method for Determination of Flutamide: Application to Protein Binding Studies

Sara Esmaeilzadeh[1,2], Hadi Valizadeh[3], Parvin Zakeri-Milani[4]*

[1] *Faculty of Pharmacy and Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran.*
[2] *Student Research Committee, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*
[3] *Drug Applied Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*
[4] *Liver and Gastrointestinal Diseases Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*

Article info
Article History:
Received: 21 April 2016
Revised: 1 June 2016
Accepted: 12 June 2016
ePublished: 30 June 2016

Keywords:
· Acetanilide
· Flutamide
· High pressure liquid chromatography
· ICH guidelines
· Protein binding

Abstract
Purpose: The main goal of this study was development of a reverse phase high performance liquid chromatography (RP-HPLC) method for flutamide quantitation which is applicable to protein binding studies.
Methods: Ultrafilteration method was used for protein binding study of flutamide. For sample analysis, flutamide was extracted by a simple and low cost extraction method using diethyl ether and then was determined by HPLC/UV. Acetanilide was used as an internal standard. The chromatographic system consisted of a reversed-phase C_8 column with C_8 pre-column, and the mobile phase of a mixture of 29% (v/v) methanol, 38% (v/v) acetonitrile and 33% (v/v) potassium dihydrogen phosphate buffer (50 mM) with pH adjusted to 3.2.
Results: Acetanilide and flutamide were eluted at 1.8 and 2.9 min, respectively. The linearity of method was confirmed in the range of 62.5-16000 ng/ml ($r^2 > 0.99$). The limit of quantification was shown to be 62.5 ng/ml. Precision and accuracy ranges found to be (0.2-1.4%, 90-105%) and (0.2-5.3 %, 86.7-98.5 %) respectively. Acetanilide and flutamide capacity factor values of 1.35 and 2.87, tailing factor values of 1.24 and 1.07 and resolution values of 1.8 and 3.22 were obtained in accordance with ICH guidelines.
Conclusion: Based on the obtained results a rapid, precise, accurate, sensitive and cost-effective analysis procedure was proposed for quantitative determination of flutamide.

Introduction

Flutamide is an anti-androgen drug which is used in Prostate cancer therapy. Its plasma peak concentration is achieved in 2 hours.[1] A single oral dose of 250 mg and 500 mg flutamide leads to maximum plasma concentration (C_{max}) of 0.02 µg/ml and 0.1 µg/ml respectively.[2] After a drug reaches to systemic circulation it can binds to plasma proteins to form drug-protein complexes. Protein binding of drugs is a reversible and dynamic process with bound and unbound drug in equilibrium. This is very important because only the unbound drug exerts pharmacological activity. In addition, changes in the plasma binding of a drug may significantly influence drug disposition. For example if a drug clearance is limited by protein binding, changes in the binding may alter clearance and also total steady-state drug concentrations.[3] Therefore it seems that protein binding studies are of great importance in clinical and pharmaceutical sciences. On the other hand analyzing the free fraction of the drug in study samples would be the main issue demanding the sensitive analytical methods.[4] The gas chromatographic determination of flutamide in blood was reported in 1988 by Schulz et al for a limited range of concentration (0.02-0.250 µg/ml).[1] In another study by Manjunath et al, HPLC system was equipped with a radioactive detector and a gradient solvent system was used for radioactive flutamide determination with 18.3 minutes retention time.[5] The HPLC analysis of flutamide in plasma were also carried out by Iwanaga et al on an separation module coupled to a dual wavelength absorbance detection system which is designed to provide the highest performance in UV-Visible detection and offers the superior sensitivity required for detection of minor impurities in complex applications. The resulting linearity range in this study was determined to be between 0.027 µg/ml and 27 µg/ml.[6] HPLC analysis of flutamide in blood serum were also reported by Filip et al with linearity range of 12.5 – 625 µg/Ml.[7] Although a HPLC/ UV method was amplified by Xu Chang-Jiang which could be employed for analysis of flutamide in blood with a good linearity area of 0.1-20 µg /ml, the method needs a very complex extraction procedure and high cost evaporation step.[8] Finally a new validated HPLC/ UV analysis for the quality

***Corresponding author:** Parvin Zakeri-Milani, Tel: +98-41-33377459, Fax: +98-41-33344798, Email: pzakeri@tbzmed.ac.ir

control of flutamide in tablets with linearity range of 0.2-25 µg/ml were developed by EL-Shahney et al.[9] However, a survey of literature reveals that available methods for flutamide analysis usually suffers from long analysis time or involve equipment not commonly available. Therefore in this research a reliable and rapid HPLC method with potential use in drug-protein binding studies was developed and validated.

Materials and Methods

Chemicals and apparatus

Flutamide was provided by Sigma-Aldrich, USA. Methanol and acetonitrile were HPLC grade and provided from Caledon, Germany. KH_2PO_4 and NaOH were supplied by Merck, Germany. Ortho-phosphoric acid was obtained from Scharlau, Spain. Acetanilide and diethyl ether were obtained from Sigma-Aldrich, USA. The HPLC grade water, generated from MilliPAK^R Express40 Milli-Q purification system by Merck, Germany was used. High performance liquid chromatography system, Knauer (Berlin, Germany) equipped with solvent delivery system consisted of an on-line degasser coupled to a quaternary low pressure gradient pump were used as chromatographic equipment.

Preparation of stock solution

Stock solution of flutamide (1600 µg/ml) in methanol was prepared and diluted with potassium dihydrogen phosphate buffer solution with pH 7.4 to obtain concentrations in the range of 0.003-16 µg/ml. Acetanilide was used as internal standard (IS). 0.9 mg /ml of acetanilide in methanol was prepared as IS stock solution and subsequently kept at 4°C. The working solution was prepared by further dilution to get the final concentration of 450 µg/ml.

Flutamide-albumin binding study

Appropriate amount of 20% human serum albumin (HSA) and flutamide solution (pH=7.4) were mixed to obtain the desired concentration of the drug (1-16 µg/ml) and HSA (4%) in 4 ml solution. Then the homogeneous mixtures were incubated in duplicate 30 minutes in a shaker incubator at 50 rpm. All samples were protected from light by wrapping the tubes in foil. After 30 minutes, the duplicate mixtures were transferred to the modified ultrafiltration systems and centrifuged at 4000 rpm for 10 minutes in temperature controlled conditions. Samples were extracted and analyzed by developed HPLC-UV method.

Extraction procedure

1ml of flutamide solution in potassium dihydrogen phosphate buffer with pH 7.4 was placed in micro tubes and spiked with acetanilide (as internal standard, 50 µl, 450 µg/ml in methanol). 400 µl of di-ethyl ether as extraction solvent was added over spiked samples in microtubes, followed by 30 seconds vortex assisted liquid-liquid micro extraction. This extraction process is needed for pre-concentration of trace levels of flutamide in samples. After centrifugation (model 5810R, Eppendorf) at 12000 rpm for 5 minutes, the organic layer was separated and evaporation was performed in a pyrex vacuum and degassing chamber resistant to pressure which was wrapped in aluminum foil to protect flutamide from light. The final residue of evaporated organic layer was dissolved in 100 µl mobile phase. Flutamide recovery in the extraction process was calculated by comparing the peak height of extracted samples without internal standard with those obtained for an equivalent amount in mobile phase directly used for analysis.

Chromatographic condition

Analysis was conducted using a Knauer HPLC system (Berlin, Germany). Analytical column was a reversed-phase C_8 column Knauer Eurosphere with 150 mm length, 4.6 mm inner diameter and particle size of 5 µm and a pre-column (Eurospher 100-5C8) at room temperature. Injection volume of 25 µl and flow rate of 1 ml/min was applied. Flutamide detection was performed by UV absorption at 226.4 nm. 29% (v/v) methanol, 38% (v/v) acetonitrile and 33% (v/v) potassium dihydrogen phosphate buffer (50 mM) with orthophosphoric acid adjusted pH of 3.2 was selected as mobile phase.

Validation of developed HPLC method

ICH guidelines concerning linearity, limits of detection and quantification, accuracy, precision, specificity and system suitability tests were used to conform developed HPLC method.[10,11]

Specificity

It is assessed by successful utilization of developed analysis approach to calculate drugs amount in their formulations or biological fluid components without any interference with their chromatograms. Therefore the specificity of the presented analysis was confirmed by comparing the chromatograms of samples containing flutamide and acetanilide with those of blank solutions.

Linearity

It relates the response directly to the analyte concentration in the sample within a fixed area. Calibration curves for flutamide were constructed between 0. 0625 and 16 µg/ml. The least-square regression analysis was used to calculate linear regression in order to assess linearity of the calibration curve.

Limits of Detection (LOD) and Quantitation (LOQ)

LOD and LOQ decide about the sensitivity of the method. LOD is the lowest concentration of the analyte detected by the method and LOQ is the minimum concentration of analyte that can be determined with acceptable precision and accuracy.[12] There are three different methods indicated in ICH guidelines. The first method relies on visual evaluation. Second method is

based on signal to noise ratio and the third method which was used in this study was based on the SD of the curve .In this method LOD and LOQ were calculated using following equations: *LOD = 3.3 SD/S* and *LOQ = 10 SD/S.*

Accuracy and Precision

Accuracy was assessed by the method of standard addition. Four standard concentrations of flutamide were prepared in triplicates. All the samples were spiked with constant amount of IS and then were assayed. %RSD and %recovery of all the concentrations were calculated. Calculation of RSD % for within- day and between-day runs leads to the determination of the precision.

System suitability tests

An essential part of a liquid chromatography technique is system suitability tests. These tests are necessary for verification of effectiveness of chromatography system for the analysis which includes column efficiency (N), selectivity factor, resolution, capacity factor and tailing factor.[13]

Capacity factor (retention factor)

The capacity factor is descript as $K'_{(A)} = (t - t_0)/ t_0$, t in this equation is the retention time of the substance and t_0 mentions retention time for un-retained compound. [14-16] Retention factor in best situation is $0.5 < K'_{(A)} < 10$.

Selectivity factor

It is the ratio of the capacity factor of two peaks. This parameter represents the ability of an HPLC method to separate two analytes from each other.

Resolution

The quality of separation between adjacent peaks is represented by resolution (R) and is descript as $R = 2(t_1-t_2)/(w_1-w_2)$ in this equation, t_1 and t_2 are the retention times and w_1 and w_2 represent the peaks width in the baseline.[15] A complete separation of peaks suggests a value of 1.5 for resolution.

Column efficiency

Column efficiency denotes the stationary phase performance and quality of the column pack. Also it is known as number of theoretical plates which can be defined as $N = 5.545[2t_R / \omega_h]^2$ where t_R and $\omega_h/2$ refer to retention time and half of the width of the peak at its base respectively.

Tailing factor

Ideal chromatographic peaks should have Gaussian-shaped but in practice most of them do not show ordinary forms. With increasing peak tailing (asymmetry) the accuracy of quantitation decreases and analysts have problems for optimum calculation of the peak area. For this reason tailing factor according to this equation: $T = \omega_{0.05} /2f$ is determined, $\omega_{0.05}$ is the interval between front and back slope of peak at 5% of its height, and f is interval between center line and front slope of peak.

Results and Discussion

Method validation

Specificity

Figure 1 shows a blank sample chromatogram and the chromatogram of a sample consisting acetanilide (IS) and flutamide. The retention times for acetanilide and flutamide were 1.8 and 2.9 min respectively. The analysis of a sample containing flutamide takes 3 minutes which allows to study a large number of samples rapidly compared to Manjunath et al developed method with retention time of 18.3 minutes.[5] As it is clear blank sample shows no peak on the chromatogram indicating no interference between component of sample matrix, acetanilide and flutamide.

Linearity

Calibration curves were plotted using the standard concentrations of 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 µg/ml of flutamide in potassium dihydrogen phosphate buffer solution with pH 7.4 after performing extraction process in duplicate for each mentioned concentration. For each concentration, the peak height was obtained and then plotted versus related concentration to achieve calibration curve. Results showed linear relationship in the range of 0.0625-16 µg/ml of flutamide on three consecutive days. This linearity range was also obtained by Xu Chang-Jiang et al for flutamide analysis in blood but very complex extraction procedure and high cost evaporation step were the main disadvantages of their work compared to ours.[8] A sample chromatogram for three concentrations is presented in Figure 1. A Linear regression was used to calculate the determination coefficient (r^2), slope and intercept of calibration curves tabulated in Table 1. Consistent with ICH guidelines, the obtained results indicate that the developed method in this work is very sensitive.[10,11] It is evident that all RSD (%) values are below the accepted value of ±15%.

Table 1. Linearity parameters for developed method in three consecutive days.

Calibration curve	Slope	Intercept	r^2
Day1	0.0742	0.0155	0.998
Day2	0.0763	0.0124	0.997
Day3	0.0744	0.016	0.988
Mean	0.0749	0.0146	0.994
RSD (%)	1.55	13.33	0.55

Accuracy and Precision

In the present research, RSD (%) and the amount of recovered flutamide by the developed analysis method were demonstrated as intra-day and inter-day precision and accuracy respectively. The intra-day precision and accuracy ranges were 0.2-1.3% and 90-105% respectively. The precision and accuracy for Inter-day

evaluation were 0.2-5.3%, 86.7-98.5% respectively. Precision RSD (%) lower than ±15% is acceptable for studied concentration within and between-day results of the precision and accuracy are exhibited in Table 2 and 3 respectively. The analysis results demonstrated a good within and between-day precision and accuracy.

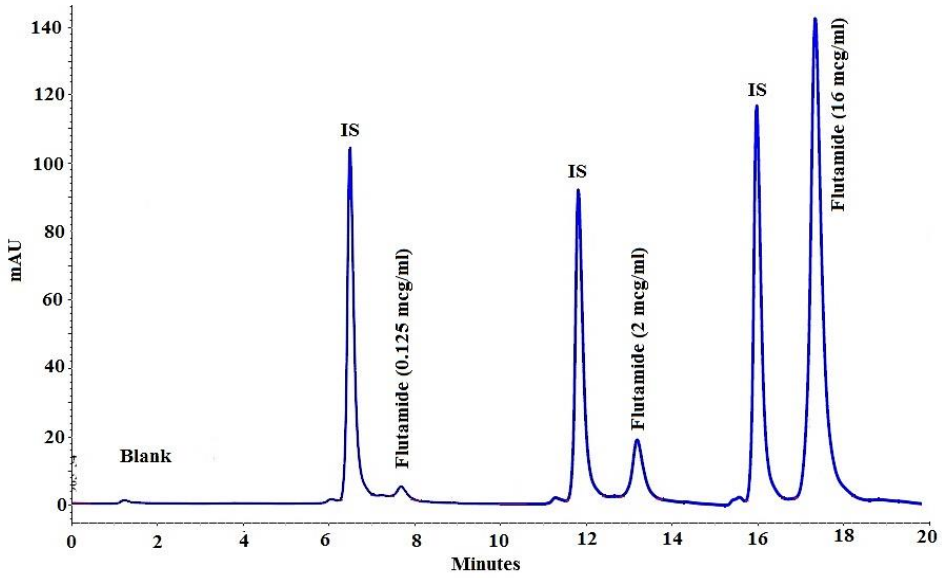

Figure 1. Representative chromatogram depicting acetanilide (IS) and flutamide peaks. Three different concentrations of flutamide in the linearity range is shown.

Table 2. Within-day precision and accuracy for developed method

Added concentration (µg/ml)	Mean measured concentration ±SD (µg/ml)	RSD (%)	Accuracy (%)
0.0625	0.059±0.0008	1.32	94.55
0.5	0.45±0.0009	0.2	90
2	2.1±0.0077	0.37	105
16	15.89±0.0370	0.233	99.34

Table 3. Between-day precision and accuracy for developed method

Added concentration (µg/ml)	Mean measured concentration ±SD (µg/ml)	RSD (%)	Accuracy (%)
0.0625	0.054±0.0029	5.3	86.7
0.5	0.44±0.0093	2.12	88
2	1.86±0.0163	0.88	93
16	15.76±0.0320	0.2	98.5

LOD and LOQ

In this study the LOD and LOQ values of 0.015 and 0.0625 µg/ml were obtained respectively. This indicates that the minimum detectable concentration of flutamide in this study is lower than LOD value of 0.05 µg/ml which was reported by Xu Chang-Jiang et al.[8] However the LOQ value of 0.027 µg/ml which was observed by Iwanaga et al in their study was lower than ours (0.0625 µg/ml). This might be explained by presence of detector with superior sensitivity than ours.[6]

System suitability tests

Retention time for un-retained compound of our analysis was 1 min. Calculated $K'_{(A)}$ values were 1.35 and 2.87 for acetanilide and flutamide respectively. The value between 0.5 and 10 for $K'_{(A)}$ shows enough space between un- retained compound and desired peak. $K'_{(A)}<1$, leads to quick elution of desired peak and not accurate retention value is obtained. $K'_{(A)}>20$ leads to elution slowly. $1< K'_{(A)}<5$ shows preferably value.[17] Therefore, retention factor values in this study are categorized in ideal range. In order to well separation of two substances, selectivity factor will be always greater

than one and in our case this value was 2.13.[17] In the present work the calculated values for resolution were 1.8 and 3.22 for acetanilide and flutamide respectively. When resolution value is equal to one, the separation of two peaks is 97.7% complete. The overlap is reduced to 0.2% when resolution value is equal to 1.5.[17] For this reason we were able to obtain chromatograms with acceptable resolution. For the column used in this study the number of theoretical plates of 5610 and 9059 were obtained for flutamide and acetanilide respectively. The sharpness of the peak is an indication of column efficiency. The higher plate number value also indicates that the chromatography column is more efficient[17] for this reason the column used had high efficiency for acetanilide and flutamide analysis. Acetanilide, flutamide tailing factors values of 1.24, 1.07 are in the acceptable range of $0.5 \leq T \leq 2$, indicating symmetry of chromatographic peaks which leads to more accurate quantitation of flutamide.[17]

Considering all the above-mentioned data, the advantages of the developed method over other works is its simplicity, applicability to wide range of concentration, very simple extraction procedure with minimum volume of extractant solvent and low cost for evaporation step.

Flutamide-Albumin Binding study

Accurate concentration of free drug in samples was determined using developed HPLC-UV analysis method. By subtracting free concentration from total concentration, the concentration of HSA-bound drug was obtained. Then the percentage of human serum albumin-flutamide binding as a function of flutamide concentration was determined. Table 4 denotes the results.

Table 4. Drug-HSA binding (%) as a function of flutamide concentration

Total drug concentration (µg/ml)	1	2	4	8	16
HSA concentration	4%	4%	4%	4%	4%
Drug-HSA binding (%)	48	78	86	88	89

It is evident that the binding increases with increasing flutamide concentration. Normally concentration-dependent protein binding could occur with all protein bound drugs. However this increase is in opposite direction which is normally described in the literature.[4,18] The presence of a competitive protein binder at lower concentrations, or multiple binding sites with different affinities could explain the observed trend.[19]

Conclusion

The proposed method for flutamide analysis in bio-fluid consist of two steps, flutamide extraction (pre-concentration) and HPLC/UV detection. The method had acceptable extraction recovery value of 100 % for flutamide and comparatively is simple and cost effective. Moreover, minimum amount of extraction solvent is used. Extraction followed by HPLC as a rapid, simple and reliable separation method, using UV detection with a good LOQ and LOD values. System suitability test parameters were in acceptable range according to ICH guidelines which make this method very suitable for kinetic in vitro analysis of flutamide in a wide range of concentration.

Acknowledgments

The authors would like to thank the authorities of the Research Center for Pharmaceutical Nanotechnology and Faculty of Pharmacy, Tabriz University of Medical Sciences, for their financial support. The results described in this paper were part of Sara Esmaeilzadeh Ph.D thesis submitted in Faculty of Pharmacy, Tabriz University of Medical Sciences. (No. 87).

Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflict of interest in this study.

References

1. Schulz M, Schmoldt A, Donn F, Becker H. The pharmacokinetics of flutamide and its major metabolites after a single oral dose and during chronic treatment. *Eur J Clin Pharmacol* 1988;34(6):633-6. doi: 10.1007/bf00615229
2. Álvarez-Lueje A, Peña C, Núñez-Vergara LJ, Squella JA. Electrochemical Study of Flutamide, an Anticancer Drug, and Its Polarographic, UV Spectrophotometric and HPLC Determination in Tablets. *Electroanal* 1998;10(15):1043-51.
3. Wood AJ, Robertson D, Robertson RM, Wilkinson GR, Wood M. Elevated plasma free drug concentrations of propranolol and diazepam during cardiac catheterization. *Circulation* 1980;62(5):1119-22. doi: 10.1161/01.cir.62.5.1119
4. Zeitlinger MA, Derendorf H, Mouton JW, Cars O, Craig WA, Andes D, et al. Protein Binding: Do We Ever Learn? *Antimicrob Agents Chemother* 2011;55(7):3067-74. doi: 10.1128/aac.01433-10
5. Shet MS, McPhaul M, Fisher CW, Stallings NR, Estabrook RW. Metabolism of the antiandrogenic drug (Flutamide) by human CYP1A2. *Drug Metab Dispos* 1997;25(11):1298-303.
6. Iwanaga T, Nakakariya M, Yabuuchi H, Maeda T, Tamai I. Involvement of bile salt export pump in flutamide-induced cholestatic hepatitis. *Biol Pharm Bull* 2007;30(4):739-44. doi: 10.1248/bpb.30.739
7. Filip M, Coman V, Avram V, Coman I. HPLC Monitoring of Flutamide Drug Used in the Prostate Cancer Treatment. 1st International Conference on Advancements of Medicine and Health Care through Technology; Sep 27-29; Cluj-Napoca, Romania 2007. p. 397-400.
8. Xu CJ, Li D. Pharmacokinetics of flutamide and its metabolite 2-hydroxyflutamide in normal and hepatic

injury rats. *Zhongguo Yao Li Xue Bao* 1998;19(1):39-43.
9. El-Shaheny RN, Yamada K. The influence of pH and temperature on the stability of flutamide. An HPLC investigation and identification of the degradation product by EI+-MS. *RSC Adv* 2015;5(5):3206-14. doi: 10.1039/c4ra13617a
10. ICH Harmonized Tripartie Guidline, Q2A. Text on Validation of Analytical Procedures. Incorporated in November 2005; http://www.ich.org.
11. ICH Harmonized Tripartie Guidline, Q2B (R1). Validation of analytical procdures: text and methodology. Incorporated in November 2005; http://www.ich.org.
12. Zakeri-Milani P, Valizadeh H, Azarmi Y, Barzegar Jalali M, Tajerzadeh H. Simultaneous determination of metoprolol, propranolol and phenol red in samples from rat in situ intestinal perfusion studies. *DARU* 2006;14(2):102-8.
13. El-Gizawy SM, Abdelmageed OH, Omar MA, Deryea SM, Abdel-Megied AM. Development and Validation of HPLC Method for Simultaneous Determination of Amlodipine, Valsartan, Hydrochlorothiazide in Dosage Form and Spiked Human Plasma. *Am J Analyt Chem* 2012;03(6):422-30. doi: 10.4236/ajac.2012.36055
14. Islambulchilar Z, Ghanbarzadeh S, Emami S, Valizadeh H, Zakeri-Milani P. Development and validation of an HPLC method for the analysis of sirolimus in drug products. *Adv Pharm Bull* 2012;2(2):135-9. doi: 10.5681/apb.2012.021
15. Zakeri-Milani P, Barzegar-Jalali M, Tajerzadeh H, Azarmi Y, Valizadeh H. Simultaneous determination of naproxen, ketoprofen and phenol red in samples from rat intestinal permeability studies: HPLC method development and validation. *J Pharm Biomed Anal* 2005;39(3-4):624-30. doi: 10.1016/j.jpba.2005.04.008
16. Watson DG. Pharmaceutical Analysis: A Textbook for Pharmacy Students and Pharmaceutical chemists. 3rd ed. Edinburgh: Elsevier Churchill Livingstone; 2012.
17. Ghoshal S. Fundamentals of Bioanalytical Techniques and Instrumentation. PHI Learning; 2009.
18. Bolandnazar S, Divsalar A, Valizadeh H, Khodaei A, Zakeri-Milani P. Development and Application of an HPLC Method for Erlotinib Protein Binding Studies. *Adv Pharm Bull* 2013;3(2):289-93. doi: 10.5681/apb.2013.047
19. Theory of Binding Data Analysis. Fluorescence Polarization Technical Resource Guide. 4th ed. Invitrogen; 2006. P. 1-18.