

Development and Application of an HPLC Method for Erlotinib Protein Binding Studies

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ABSTRACT

Purpose: The aim of the present study was to develop a simple and rapid reversed-phase high performance liquid chromatographic method with UV detection for erlotinib hydrochloride quantification, which is applicable for protein binding studies. **Methods:** Ultrafiltration method was used for protein binding study of erlotinib hydrochloride. For sample analysis a simple and rapid reversed-phase high performance liquid chromatographic method with UV detection at 332 nm was developed. The mobile phase was a mixture of methanol, acetonitril and potassium dihydrogen phosphate buffer (15:45:40 %v/v) set at flow rate of 1.3 ml/min. **Results:** The run time for erlotinib hydrochloride was approximately 6 minutes. The calibration curve was linear over the range of 320-20000 ng/ml with acceptable intra- and inter-day precision and accuracy. The intra-day and inter-day precisions were less than 10% and the accuracies of intra and inter-day assays were within the range of 97.20-104.83% and 98.8-102.2% respectively. **Conclusion:** Based on the obtained results, a simple, accurate and precise reversed-phase isocratic HPLC method with UV detection has been optimized and validated for the determination of erlotinib hydrochloride in biological samples.

Introduction

Lung cancer is the leading cause of cancer mortality in the world.¹⁻³ The epidermal growth factor receptor (EGFR) is mutated and over expressed in many human cancers, such as head and neck, breast, ovarian and non-small cell lung cancers (NSCLC).⁴ Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) are used as first-line and efficient therapy in the treatment of advanced non-small cell lung cancer patients.^{2,5} The family of epidermal growth factor receptor (HER1/EGFR), containing four members, are essential in modulating cell proliferation, cell differentiation, and cell survival in many tissue types.⁶ Erlotinib is a quinazolinamine with the chemical name of N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine.⁷ The chemical structure of erlotinib is shown in Figure 1.

Plasma protein binding of erlotinib is reported to be approximately 93% and it has an apparent volume of distribution of 232 liters.^{3,8,9} It is a white powder, slightly soluble in water, soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide

(DMF), which should be purged with an inert gas.¹⁰ Erlotinib is almost a new drug used for the treatment of non-small cell lung cancer after the failure of more than one or two courses of previous chemotherapy. It is an orally available low molecular weight EGFR inhibitor that binds competitively to the ATP binding site at the kinase domain of EGFR and inhibits EGFR tyrosine kinase autophosphorylation by inhibition of the intracellular domain.^{3,6} Erlotinib is metabolised by the hepatic cytochromes in humans, primarily CYP3A4/CYP3A5 and to a lesser extent by CYP1A2 and the pulmonary isoform CYP1A1.^{11,12} On the other hand, binding of drugs to plasma and tissue proteins significantly affects the pharmacokinetic and pharmacodynamic behaviors of drugs. The pharmacologic effect of a drug in the body is related to the free drug concentration at the target site. Because the bound drug is kept in the blood stream while the unbound components of the drug, may be distributed or metabolized, making them the active part of the drug. Therefore protein binding studies would be of great

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importance in clinical and pharmaceutical sciences. For this purpose drug analysis in study samples is the main issue. Although several HPLC techniques have been developed for the assay of erlotinib, like liquid chromatography – tandem mass spectrometry (LC/MS/MS) methods, there is still no simple and rapid HPLC method available for its quantification in clinical and pharmaceutical samples.¹³⁻¹⁸ Therefore in the present study a simple, sensitive, rapid and effective HPLC method was developed and validated for quantification of erlotinib hydrochloride with the application to protein binding studies.

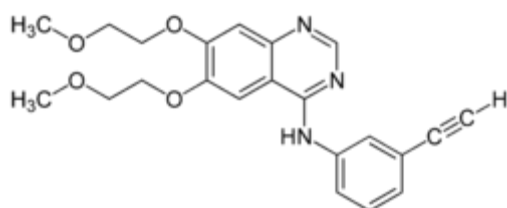


Figure 1. Chemical structure of Erlotinib.

Materials and Methods

Chemicals

Dialysis membranes and dialyzers were obtained from Harvard (Harvard, USA). HPLC grade solvents, such as methanol and acetonitril were purchased from Merck (Darmstadt, Germany). Triethylamine, potassium dihydrogen phosphate, sodium hydroxide, and orthophosphoric acid were also provided from Merck (Darmstadt, Germany). Pharmaceutical-grade human albumin 20% was purchased from CSL Behring GmbH, Germany. Double-distilled water was used during the experiments.

Chromatographic system

Analysis was performed with a knauer high performance liquid chromatography system (Berlin, Germany). Analytical column used for analysis was a reversed-phase Symmetry C18 column shimadzu, Shim-pack VP ODS (250 mm × 4.6 mm, 5 μm) at room temperature. Injection volume was 20 μl which was injected into the column using a Hamilton (Bonaduz, Switzerland) injector syringe and the flow rate was set at 1.3 ml/min. Erlotinib hydrochloride was detected by UV absorption at 332 nm. The mobile phase was a mixture of 15% (v/v) methanol, 45% (v/v) acetonitril and 40% (v/v) potassium dihydrogen phosphate buffer with pH adjusted to 4.5 with orthophosphoric acid.

Preparation of stock and standard solutions

The stock solution of erlotinib was prepared by dissolving 10 mg erlotinib hydrochloride in potassium phosphate buffer, adjusted to pH 7.4 with NaOH 0.2 M, in a 50 ml volumetric flask to provide a concentration of 200 μg/ml. Then, working standard solutions were prepared by serial dilution using potassium phosphate buffer (pH 7.4) solution to obtain erlotinib

hydrochloride concentrations of 0.3, 0.62, 1.25, 2.5, 5, 10 and 20 μg/ml for demonstration of calibration curve.

Method Validation

Validation of the assay consisting of linearity, lower limit of detection and quantitation (LOD and LOQ), intra-day and inter-day accuracy and precision of the method was performed. To assess linearity, known concentrations of erlotinib hydrochloride in PBS buffer were prepared. The accuracy and precision data were obtained by analyzing four aliquots of samples at different concentrations. Intraday reproducibility was determined using four aliquots of samples and inter-day reproducibility was determined over a 3- day period (n=4). Accuracy was evaluated by calculating the percentage deviation of the calculated concentration and the theoretical concentration while the precision was assessed via calculating the coefficient of variation percentage (CV %) for intra- and inter-day runs. The acceptable value for accuracy is less than ±15% deviation from the nominal values and, CV% ≤ ±15% for precision.¹⁹ There are three different methods for determination of LOD and LOQ as indicated in ICH guidelines. However in this study, to determine the LOD, the signal to noise ratio was used by comparing test results from samples with known concentrations to blank samples. The LOQ is also defined as the lowest concentration that can be quantitate with acceptable precision and accuracy under the stated experimental condition.^{20,21}

Stability test

The stability of working standard samples was determined at ambient temperature by analyzing the solutions over a period of 8 hours (considering the approximate time required for preparation of samples and analysis by HPLC method). The aim was to ensure that the preparation of samples and analysis time did not contribute to the degradation of drug to indicate that the samples will remain stable during the course of the analysis.^{19,21,22}

Protein binding study

Ultrafiltration (UF) method which is a simple and reliable procedure for measuring the protein unbound fraction of a drug in plasma was used in this investigation. Aqueous solutions containing 2, 4, 6, 8, 10 μg/ml of erlotinib hydrochloride and 0.04 g/ml of human serum albumin were prepared. After a period of 1 hour, to produce ultrafiltrates containing unbound drug, the samples were added in the ultrafiltration system (cellulose acetate membrane with 25KDa molecular weight cut-off). Centrifugations were performed at 37°C, for 10 min at 4000 rpm, resulting in filtrate volumes of 0.3–0.4 mL. The fraction of unbound drug was calculated as the ratio of the ultrafiltrate (free) concentration and the total concentration determined in the HPLC analysis. The drug concentrations were plotted according to

Scatchard where the abscissa represents the binding r (the number of molecules of drug bound per molecule of albumin), and the ordinate r/D_f (D_f is free drug concentration). The data of r and D_f were fitted by linear least squares regression analysis. Then the number of binding sites and association constants were calculated.²³

Results and Discussion

Linearity

Six standard samples (0.3, 0.6, 1.2, 2.5, 5, 10 and 20 µg/ml) were prepared to generate the calibration curve

for linearity of the method. Representative chromatogram of consecutively concentrations of erlotinib hydrochloride is shown in Figure 2. Statistical analysis using least square regression indicated excellent linearity for erlotinib hydrochloride in the mentioned range. A good correlation between erlotinib hydrochloride peak heights and drug concentration was observed with $r^2 \geq 0.99$ for all standard curves (Table 1). Concentration curves for erlotinib hydrochloride had a mean slope, intercept and r^2 of 0.33, -22.8 and 0.996 respectively. The retention time was approximately 6 min.

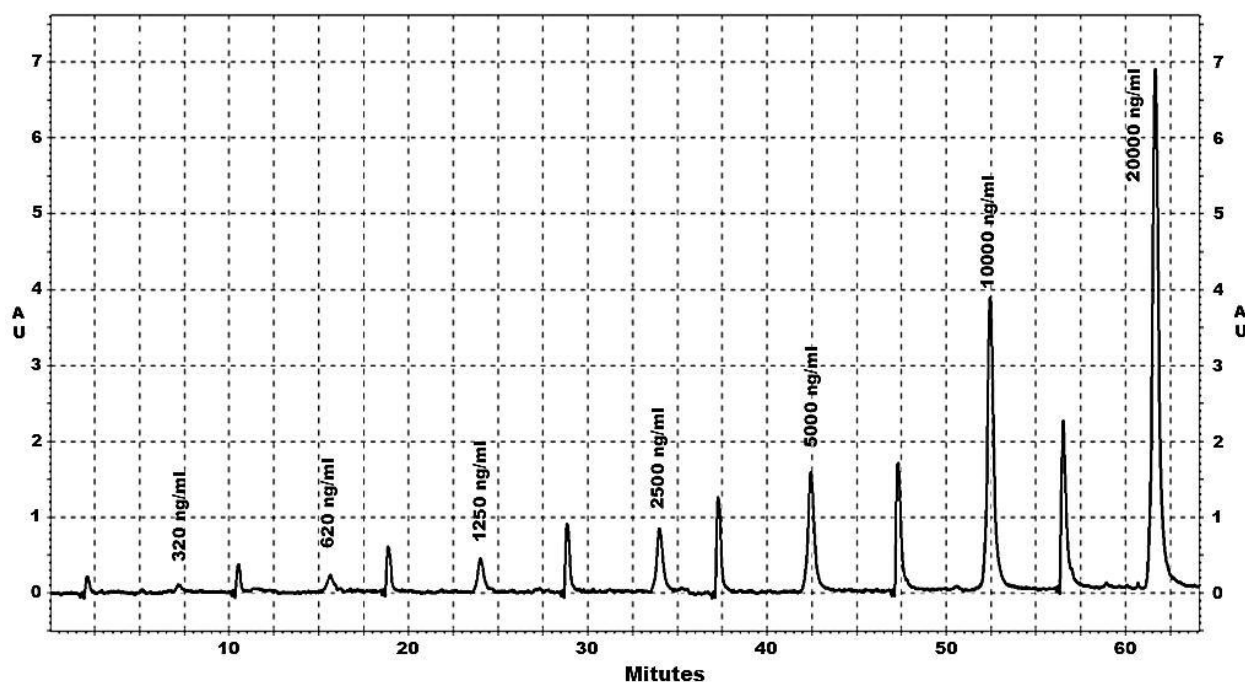


Figure 2. Representative chromatogram of erlotinib hydrochloride standard samples with different concentrations.

Table 1. Analytical parameters of erlotinib hydrochloride calibration curves on three consecutive days.

Standard curve	Slope	Intercept	r^2
Day 1	8861.0	2053.0	0.9979
Day 2	9010.6	1776.1	0.9970
Day 3	9019.2	1343.0	0.9976
Mean	8963.6	1724.0	0.9970
RSD	0.009	0.207	0.0004

LOD and LOQ

The LOD was defined as the analyte concentration that gives a signal equal to $y_b + 3.3s_b$, where y_b is the signal of the blank and s_b is its standard deviation. Similarly, the LOQ was defined as $y_b + 10s_b$. In the unweighed least-squares method it is quite suitable in practice to use s_{xy}

(Residual standard deviation) instead of s_b and the value of the calculated intercept instead of y_b . Thus $LOD = 3.3 s_{xy} / b$ and $LOQ = 10 s_{xy} / b$, Where b is the slope of the regression line. Based on the above equations, the calculated LOD and LOQ values for erlotinib hydrochloride were 46 and 150 ng/ml respectively.

Accuracy and Precision

The intra- and inter-day precision and accuracy was shown as percent of coefficient of variation (CV %) and mean percentage of analyte recovered in the assay, respectively. The Intra-day precision, accuracy and relative errors range were calculated to be 1.91-3.07%, 97.2-104.83% and 0.6- 4.83, respectively. The same parameters for inter-day evaluations were 1.56-9.91%, 98.8-100% and 0-2.2%, respectively. Precision (CV %) and relative error percent acquired at all of concentrations do not exceed $\pm 15\%$ (Table 2) which is required by guidelines.

Table 2. Intra-day and inter-day accuracy and precision obtained from calibration curves with four levels of QC samples.

Added concentration (µg/ml)	Intra-day				Inter-day			
	Measured concentration (mean ± SD, µg /ml)	Precision (CV %)	Accuracy (%)	Relative Error %	Measured concentration (mean ± SD, µg /ml)	Precision (CV %)	Accuracy (%)	Relative Error %
0.62	0.65±0.02	3.07	104.83	4.83	0.62±0.05	8.06	100	0.0
2.5	2.43±0.19	7.81	97.2	2.8	2.47±0.24	9.91	98.8	1.2
10	9.93±0.26	2.61	99.3	0.7	10.22±0.16	1.56	102.2	2.2
20	19.88±0.38	1.91	99.4	0.6	19.92±0.56	2.81	99.6	0.4

Stability

The stability of erlotinib hydrochloride standard samples was tested at room temperature by testing the solutions over a period of 8 hours (Table 3). The purpose of this test was to confirm that erlotinib hydrochloride in both standard and protein binding samples were stable during the course of analysis. The solutions were considered stable if the variability in the assay results was less than 15 % of initial. Results indicated that erlotinib hydrochloride was quite stable during sample preparation and analysis period.

Table 3. Erlotinib Stability during 8h at ambient temperature.

Added concentration (µg/ml)	Measured concentration (mean ± SD, µg /ml)	Standard Deviation (%)
0.62	0.627±0.02	0.04
5	4.99±0.05	0.05
20	19.55±0.12	0.20

Protein binding studies

The free drug concentrations in ultrafiltrates and mean protein binding percentages were in the range of 0.027-0.26 µg/ml and 95.6-98.6% respectively. v , the number of binding sites and K , the association constant was calculated according to the regression line equation as 0.0328 and 5×10^6 respectively (Figure 3). Based on the results, the protein binding percentage of erlotinib hydrochloride reduced with increasing the drug concentration. Concentration-dependent protein binding has been observed for many drugs such as some macrolides and beta-lactams. As the concentration of drug increases, binding sites on proteins are increasingly saturated, resulting in higher percentages of unbound drug.²³

Conclusion

The mentioned method described in this paper has acceptable linearity, accuracy, and precision. Therefore the validated method can be used for routine analysis of

erlotinib hydrochloride in samples of different studies like plasma protein binding investigations which are of great importance in clinical and pharmaceutical sciences.

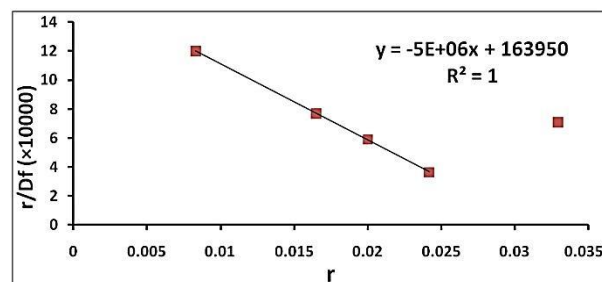


Figure 3. Scatchard plot of erlotinib.

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Conflict of Interest

The authors report no conflict of interest in this study.

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