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SHORT COMMUNICATION

Development and Validation of a Spectrofluorimetric Method for the Determination of Erlotinib in Spiked Human Plasma

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Abstract A rapid and sensitive spectrofluorimetric method was developed and validated for the determination of erlotinib (ETB), a potent anticancer drug, in spiked human plasma without any derivatization. The described method was validated and the analytical parameters of linearity, accuracy, precision (intra- and inter-day), limit of detection (LOD), and limit of quantification (LOQ) were evaluated. The relation between the fluorescence intensity and concentration was found to be linear (r^2 0.9998) over the range 125 to 1000 ng/mL with the detection limit of 15 ng/mL. A simple liquid-liquid extraction method was followed in order to extract the drug from spiked plasma. The mean absolute recoveries of ETB were 85.59 % (±0.57), 86.91 % (±1.77) and 89.31 % (±3.01) at spiked plasma ETB concentration of 5000, 3750 and 2500 ng/mL, respectively. The spectrofluorimetric method presented here is a rapid, simple, specific, and reproducible method and can be used to characterize the plasma pharmacokinetics of ETB.

Keywords Erlotinib · Spectrofluorimetric · Spiked human plasma · Validation

Introduction

The epidermal growth factor receptor (EGFR) tyrosine kinase (TK) is recognized as an important molecular target expressed in various types of solid tumors [1]. Erlotinib, N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine,(ETB) is a highly selective and potent inhibitor of EGFR TK [2]. It is clinically used for the treatment of several advanced malignancies including non-small cell lung cancer (NSCLC), pancreatic, ovarian, head and neck, breast, prostate, colorectal, hepatic, and renal cancers [3].

Several techniques such as high performance liquid chromatography with tandem mass spectrometry (LC-MS) [4,5] or UV visible spectrophotometry [6,7], hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC-MS/MS) [8] have been reported for the determination of ETB in human plasma. However, these techniques are time-consuming, complex, expensive and require trained personnel for clinical analysis of plasma samples.

A preliminary study in our lab showed the intrinsic fluorescence property of ETB. Further evaluation of this phenomenon led to the hypothesis that the application of fluorescence spectrometry could be one of the viable alternatives for the determination of ETB in human plasma. Fluorescence spectrometry is a very simple, rapid, efficient, selective, and highly sensitive technique for determination of drug in plasma [9–12]. A thorough survey of the literature on bio-analytical methods for ETB reveals a lack of supporting information employing spectrofluorimetric method for the analysis of ETB in biological fluids. Although a study was published concerning the application of fluorescence spectroscopy and UV-visible spectrometry to investigate the binding ability of ETB with bovine serum albumin [13], it did not report bio-analytical assay of ETB in plasma. Therefore, the aim of the current study is to develop and validate a simple and rapid spectrofluorimetric method for the *in vitro* determination of ETB in spiked human plasma. This method does not require derivatization of the drug due to the intrinsic fluorescent activity of ETB. Results from this

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study suggest that this method can afford a rapid, simple, accurate and sensitive technique to determine ETB in plasma.

Experimental

Chemicals and Reagents

ETB, free base (98 % purity) was purchased from Cayman Chemical (MI, USA). Glycine, analytical grade and sodium hydroxide, analytical grade were supplied by Fisher (NJ, USA). All solvents (water, HPLC grade; acetonitrile, HPLC grade; hexane, HPLC grade and ethyl acetate, ACS grade) were also supplied by Fisher (NJ, USA). Drug free human plasma was obtained from Innovative Research (MI, USA) and stored at −80 °C until analysis.

Instruments

All fluorescent measurements were performed using a Hitachi F-2500 fluorescence spectrophotometer (Tokyo, Japan) equipped with xenon lamp. Slit widths for both excitation and emission monochromators were set at 5 nm and all measurements were made in quartz cells with path length of 1.0×1.0 cm. Thermo scientific IEC CL31R multispeed centrifuge (NC, USA) with Eppendorf rotor (F16-48×1.5/2.0, Fibrolite®, Piramoon Technol. Inc., CA, USA) was used to separate insoluble components.

Standards and Sample Preparations

An accurately weighed sample (20.00 mg) of ETB was transferred into a 100 mL volumetric flask, dissolved in about 80 mL of acetonitrile and made up to the volume with acetonitrile to prepare standard ETB stock solution. The ETB stock solution was found to be stable for at least 30 days. Working solutions with concentrations ranging from 125 to 1000 ng/mL were made by appropriate serial dilution with acetonitrile.

Table 1 Validation data for analysis of ETB using spectrofluorimetry

Parameter	Result
Linearity range (ng/mL)	125.00–1000.00
λ_{ex}/ λ_{em} (nm)	247/470
Regression equation	I_F=8.8922 C–91.6566
Correlation coefficient (r^2)	0.9998
Slope ± SD	8.8922±0.0331
Standard error of slope	0.019
Intercept ± SD	91.6566±44.6161
Standard error of intercept	25.7591
Lower limit of detection	15.0523
Lower limit of quantification	50.1744

λ_{ex} maximum wavelength of excitation; λ_{em} maximum wavelength of emission; I_F corrected fluorescence intensity; C concentration; SD standard deviation

Procedure for Calibration Curve

The ETB stock solution was diluted with acetonitrile to obtain standard solutions of concentration ranging from 125 to 1000 ng/mL. The fluorescence intensity of the standard solutions was measured at 470 nm following an excitation at 247 nm (Fig. 1). The intensity of the blank solution without ETB was measured. The corrected fluorescence intensity (actual intensity less blank intensity) was plotted against the corresponding drug concentrations to obtain the calibration curve. The

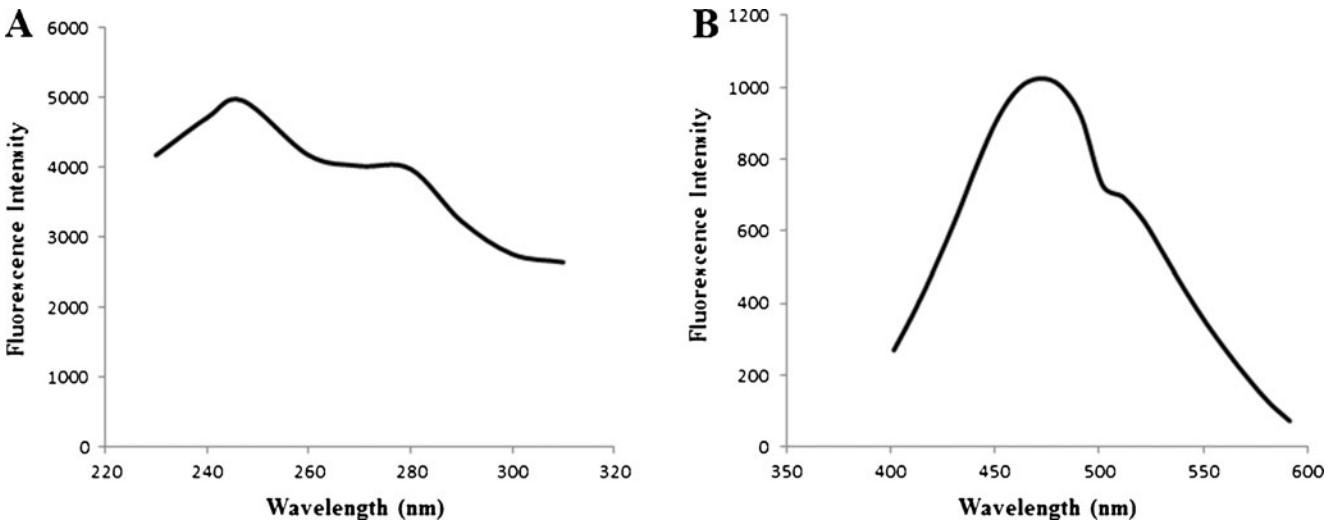

Fig. 1 Excitation (**a**) and emission (**b**) spectrum of ETB in acetonitrile at room temperature

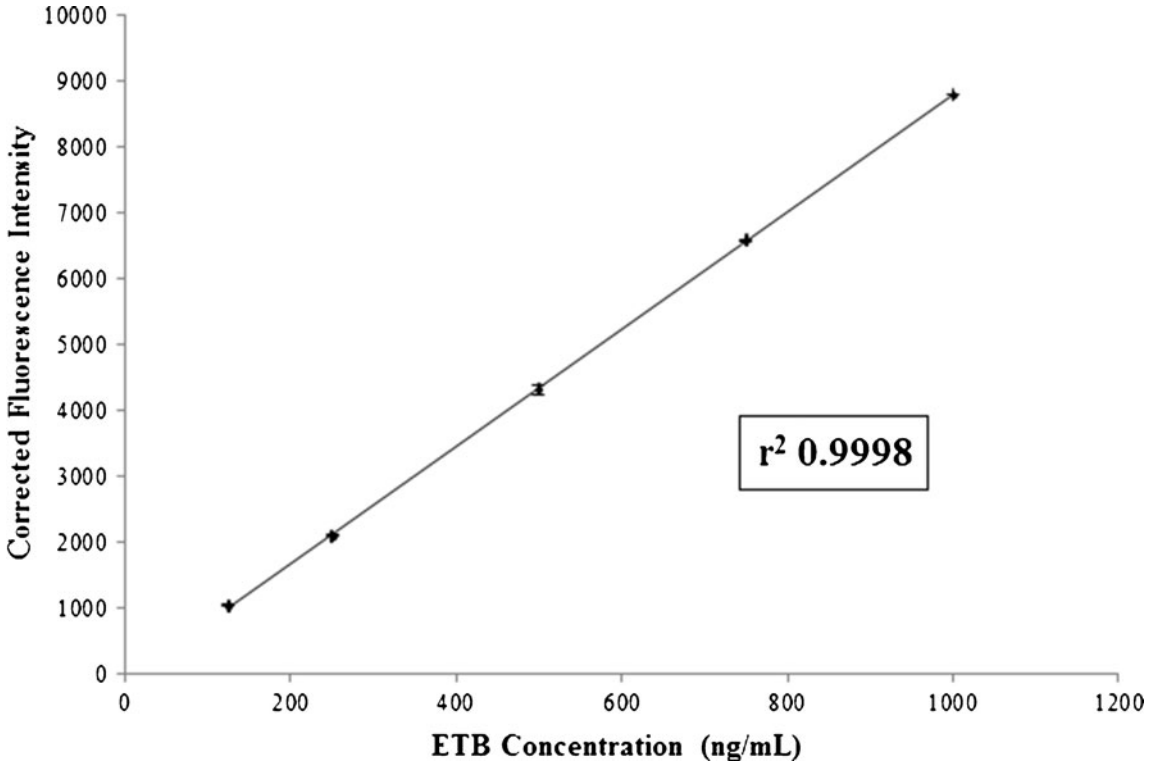

Fig. 2 Calibration curve of ETB in acetonitrile

corresponding regression equation was derived to validate the method.

Method Validation

The validation of the method was carried out by establishing linearity, accuracy, precision (intra- and inter-day), limit of detection (LOD), and limit of quantification (LOQ).

Procedure for Extraction of Drug from Spiked Human Plasma

The extraction protocol was based on liquid-liquid extraction, adapted from published work by Masters *et al* [4]. Briefly, frozen human plasma samples (stored in a −80 °C freezer) were thawed to ambient temperature. A 0.1 mL aliquot of the human plasma was placed in a 1.5 mL

Table 2 Accuracy of the spectrofluorimetric method for determining ETB

Days	Actual Conc. (ng/mL)	Mean Conc. (ng/mL)	± SD	% Nominal	% RSD	% Bias	SEM
1	125	131.61	0.35	105.29	0.26	5.29	0.20
2	125	133.12	0.06	106.50	0.05	6.50	0.04
3	125	133.23	0.17	106.58	0.13	6.58	0.10
1	250	250.02	0.24	100.01	0.10	0.01	0.14
2	250	252.38	0.23	100.95	0.09	0.95	0.13
3	250	252.20	0.43	100.88	0.17	0.88	0.25
1	500	508.10	2.60	101.62	0.51	1.62	1.50
2	500	493.44	0.72	98.69	0.15	−1.31	0.41
3	500	501.63	1.58	100.33	0.31	0.33	0.91
1	750	756.07	0.40	100.81	0.05	0.81	0.23
2	750	757.35	1.30	100.98	0.17	0.98	0.75
3	750	756.70	0.62	100.89	0.08	0.89	0.36
1	1000	1003.13	1.39	100.31	0.14	0.31	0.80
2	1000	1005.24	1.14	100.52	0.11	0.52	0.66
3	1000	1005.09	0.58	100.51	0.06	0.51	0.33

SD standard deviation; *SEM* standard error of mean; *RSD* relative standard deviation; 1,2,3 represents measurements obtained on day 1, day 2 and day 3 respectively (n=3 for each day)

polypropylene microcentrifuge tube. A volume of 0.04 mL of standard drug solutions (at three different concentrations of 17500, 13125 and 8750 ng/mL of ETB) was added to the blank human plasma to achieve spiked plasma concentrations of 5000 ng/mL, 3750 ng/mL and 2500 ng/mL, respectively. The individual tubes were vortexed for 10 seconds and incubated at room temperature (20–25 °C) for 5 min following which a 0.1 mL portion of 100 mM NaOH/glycine pH 12 buffer was added. The tube was then mixed for 10 s on a vortex mixer. Next, a volume of 1 mL of hexane: ethyl acetate (50:50 v/v) was added to the tube and mixed vigorously for 20 seconds on a vortex mixer. The samples were then centrifuged at 9800 rpm, 4 °C for 15 min to assure phase separation. The resulting organic layer was transferred to a 1.5 mL polypropylene microcentrifuge tube and evaporated to dryness using nitrogen purging. Acetonitrile (1 mL) was added to the tube, mixed for 10 seconds on the vortex mixer and analyzed in spectrofluorimeter. A blank solution was prepared in a similar manner using 0.1 mL of human plasma but without addition of ETB.

Results and Discussion

The calibration data with parameters for the analytical performance of the proposed method are summarized in Table 1. For evaluation of linearity at the selected conditions, determination of ETB was carried out at five concentration levels (n=3), respectively. The calibration curves of ETB were linear over the concentration range of 125–1000 ng/mL with good correlation of coefficient (r^2) of 0.9998 (Fig. 2). The LOD and LOQ were calculated employing the following formula [9];

$$LOD = \frac{3S_b}{m}$$

$$LOQ = \frac{10S_b}{m}$$

where S_b is the standard deviation of the intercept of regression line, and m is the slope of the calibration curve. On this basis, the LOD and LOQ of the proposed method for standard ETB solution were 15.05 and 50.17 ng/mL, respectively. LOQ was found to be clinically relevant for the quantification of ETB in plasma. Phase I studies reported a $C_{ss,\ min}$ of 1200± 620 ng/ml and 1642±1085 ng/ml in NSCLC patients treated with the recommended 150 mg daily dose, in western and Japanese patients, respectively[14] . Overall, the reported data support that our LOQs are sufficient to quantify plasma ETB $C_{ss,\ min}$ in NSCLC patients.

Accuracy, intra-day and inter-day precisions of the method were determined (shown in Tables 2 and 3). Three replicate samples in the same day, as well as on three consecutive days were assayed for intra-day and inter-day precision at five different concentrations. Accuracy was calculated as % bias using the following equation,

$$\%Bias = \frac{(Nominal\ ETB\ concentration - measured\ ETB\ mean\ concentration)*100}{Nominal\ ETB\ concentration}$$

The % bias was found to be ranged from −1.31 to 6.58 %, indicating the accuracy of the method. The intra-day and inter-day precisions expressed as the % relative standard deviation (% RSD) for ETB ranged from 0.05 to 0.51 % and 0.11 to 1.95 %, respectively. The low % RSD indicates the inter-day and intra-day precision of the method. Hence, these results indicate that the proposed spectrofluoremetric method is accurate and precise.

Recovery studies were carried, by spiking varying quantities of pure drug solutions to the human plasma. The solvent mixture of hexane/ethyl acetate (50:50 v/v) with NaOH/glycine pH 12 buffer, selected for the liquid-liquid

Table 4 Accuracy and recovery data of ETB in spiked human plasma

Spiked (ng/mL)	Found (ng/mL)	Accuracy %	Mean Recovery %	±SD	% RSD	SEM
700	613.13	−12.41	85.59	0.57	0.66	0.33
525	456.28	−13.09	86.91	1.77	2.04	1.03
350	312.59	−10.69	89.31	3.01	3.36	1.74

Table 3 Intra- and inter-day precision of the proposed method

Actual Conc (ng/mL)	Repeatability (intra-day precision)			Intermediate Precision (inter-day)		
	Mean Conc. ± SD (n=3)	SEM	% RSD	Mean Conc. ± SD (n=9)	SEM	% RSD
125	131.72±0.34	0.19	0.26	128.49±2.51	0.83	1.95
250	249.60±0.23	0.13	0.09	247.37±1.77	0.59	0.72
500	506.72±2.59	1.49	0.51	496.88±8.44	2.81	1.69
750	753.75±0.39	0.23	0.05	751.94±1.59	0.53	0.21
1000	999.88±1.38	0.79	0.14	1000.34±1.15	0.38	0.11

extraction method, showed acceptable recoveries. The mean absolute recoveries of ETB were 85.59 % (±0.57), 86.91 % (±1.77) and 89.31 % (±3.01) at spiked plasma ETB concentration of 5000, 3750 and 2500 ng/mL, respectively. The three spiked plasma concentrations fall within the steady state plasma concentration range found in patients [15,16]. The recovery values are in good agreement with the findings of Faivre et al. [17] concerning HPLC-UV method for quantification of ETB in human plasma. The results reported in Table 4 reveal that the % RSD and percent mean of extraction recovery for spiked plasma samples are in the range of 0.66–3.36 %, 85.59–89.31 %, respectively. Relatively, high plasma protein binding (90–95 % in humans) could be responsible for low recovery values (below 90 %) [18].

Conclusion

A sensitive and rapid method for the determination of ETB in spiked human plasma is reported using spectrofluorimetry based on the intrinsic fluorescence properties of ETB. The proposed method was optimized and validated for linearity, precision and accuracy. The results of method validation indicates the linearity over the range from 125 to 1000 ng/mL (r^2=0.9998) with a detection limit of 15 ng/mL, which is well within the observed therapeutic plasma levels for ETB. The overall extraction efficiency was greater than 87 % for spiked plasma samples based on simple liquid-liquid extraction method. Major advantages of this method are simple sample preparation, low sample volume (0.1 mL), and high sample throughput.

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