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Research Article

Quantitative determination of erlotinib in human serum using competitive enzyme-linked immunosorbent assay

Yuta Yamamoto, Tetsuya Saita *, Yutaro Yamamoto, Masashi Shin

Applied Life Science Department, Faculty of Biotechnology and Life Science, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

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ABSTRACT

A selective and sensitive competitive enzyme-linked immunosorbent assay (ELISA) method was developed and validated for the quantification of erlotinib in 50 μL of samples of human serum. Anti-erlotinib serum was obtained by immunizing mice with an antigen conjugated with bovine serum albumin and 3,4-bis(2-methoxyethoxy)benzoic acid using the *N*-succinimidyl ester method. Enzyme labeling of erlotinib with horseradish peroxidase was similarly performed using 3,4-bis(2-methoxyethoxy)benzoic acid. A simple competitive ELISA for erlotinib was developed using the principle of direct competition between erlotinib and the enzyme marker for anti-erlotinib antibody, which had been immobilized on the plastic surface of a microtiter plate. Serum erlotinib concentrations lower than 40 ng/mL were reproducibly measurable using the ELISA. This ELISA was specific to erlotinib and showed very slight cross-reactivity (6.7%) with a major metabolite, *O*-desmethyl erlotinib. Using this assay, drug levels were easily measured in the blood of mice after oral administration of erlotinib at a single dose of 30 mg/kg. ELISA should be used as a valuable tool for therapeutic drug monitoring and in pharmacokinetic studies of erlotinib.

1. Introduction

Erlotinib (Fig. 1), an epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), is widely used to treat non-small cell lung cancer and unresectable pancreatic cancer [1,2]. However, it is associated with a higher incidence of interstitial lung disease than other anticancer drugs, and many fatal cases have been reported [3]. It also frequently causes acneiform rashes by inhibiting the expression of EGFR in the stratum basale and skin appendages. These reactions are frequently reported as a reason for dose reduction or interruption of treatment in patients receiving erlotinib [4,5].

Erlotinib is metabolized by CYP3A4, CYP1A2, and CYP3A5 in the liver, and the induction of CYP3A4 via tobacco smoking has been shown to decrease the blood concentration of erlotinib by about 50% [6]. Food also influences the absorption of erlotinib. Taking erlotinib after consuming a high-fat, high-calorie meal has been shown to increase maximum blood concentrations by about 1.5-fold and the AUC about 2-fold [7]. The severity of skin damage caused by erlotinib has been shown to be correlated with the concentration of erlotinib in the blood [8], which indicates that therapeutic drug monitoring (TDM) is needed to minimize the toxicity of erlotinib and improve the response to treatment.

O-Desmethyl erlotinib is known as a major metabolite in humans (Fig. 1) [9]. During long-term therapy, the plasma concentration of *O*-desmethyl erlotinib is about 10% of that of erlotinib [10]. Therefore, an analytical method specific to erlotinib must be developed for TDM or pharmacokinetic studies. Previous TDM and pharmacokinetic studies of erlotinib have used high-performance liquid chromatography (HPLC) [11] and liquid chromatography with tandem mass spectrometry (LC–MS/MS) [12–17]. However, no immunoassay technique that compares favorably with these analytical methods has been developed. The reason for this is that it is very challenging to produce an anti-erlotinib antibody that is not cross-reactive with *O*-desmethyl erlotinib, the major metabolite of erlotinib. We previously created a specific antibody, which is not cross-reactive with the major metabolite, and changed part of the structure of the drug to a hapten antigenic structure, by considering the structure of the major metabolite, to develop an enzyme immunoassay technique that could be used for pharmacokinetic studies of the antibody [18–20]. Similarly, we created a specific anti-erlotinib antibody by changing part of the structure of erlotinib to a hapten antigenic structure to develop enzyme immunoassay techniques that could be used for pharmacokinetic studies on erlotinib.

In this study, we successfully developed the first specific and sensitive competitive enzyme-linked immunosorbent assay (ELISA) for erlotinib using a polyclonal antibody against part of the structure of erlotinib and herein report the technique. The initial application of the assay for the measurement of erlotinib

* Corresponding author.
E-mail address: sait1102@life.sojo-u.ac.jp (T. Saita).

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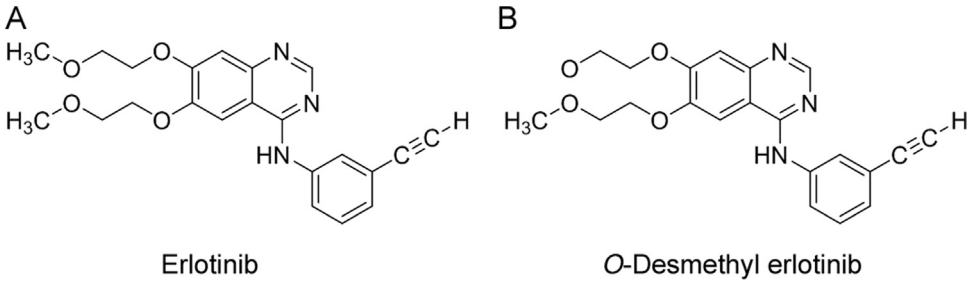

Fig. 1. Chemical structures of erlotinib and its major metabolite.

levels in mice demonstrates its usefulness for the assessment of basic pharmacokinetic parameters.

2. Materials and methods

2.1. Chemicals and reagents

Erlotinib hydrochloride and *O*-desmethyl erlotinib were obtained from AdooQ BioScience LLC (Irvine, CA, USA). Ethyl 3,4-bis(2-methoxyethoxy)benzoate (EBMB) was obtained from AK Scientific, Inc (Union City, CA, USA). 2,4,6-Trinitrobenzene sulfonic acid was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Horseradish peroxidase (HRP) and 3,3,5,5-tetramethylbenzidine (TMB) were obtained from Boehringer Ingelheim Pharma GmbH (Ingelheim, Germany). Sodium hydroxide, hydrochloric acid, sulfuric acid, sodium dihydrogenphosphate dehydrate, disodium hydrogenphosphate 12-water, sodium azide, tris(hydroxymethyl)aminomethane (Tris), 1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide, dioxane, ethyl acetate, *N,N*-dimethylformamide and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydrogen peroxide (30% in water) was obtained from Nacalai Tesque Inc. (Kyoto, Japan).

2.2. Preparation of the immunogen for erlotinib

The erlotinib immunogen was prepared with part of the structure of erlotinib (EBMB) as shown in Fig. 2. EBMB (10 mg, 33.5 μmol) was dissolved in 500 μL of 1 M NaOH and the resulting solution was left to stand at 60 °C for 1 h. The resulting EBMB carboxylate was acidified by the addition of 550 μL of 1 M HCl and then extracted with ethyl acetate. The organic layer was separated and evaporated under reduced pressure. The residue was dissolved in 95% dioxane (1 mL). EDC (12.7 mg, 67 μmol) and N-hydroxysuccinimide (7.7 mg, 67 μmol) were added to the dioxane solution, and the solution was left to stand at room temperature for 2 h. The reaction mixture containing succinimidyl EBMB was immediately mixed with BSA (20 mg) in 1 mL of 0.1 M phosphate buffer (pH 7.0) and incubated at room temperature for 2 h. The reaction solution was dialyzed in 1 mM phosphate buffer (pH 7.0) for 24 h. The purified conjugate was lyophilized and used as an immunogen for erlotinib. The trinitrobenzene sulfonic acid method was used to determine the primary amine [21], and about 18.1 EBMB molecules were found to be coupled with each molecule of BSA based on the reduction of the primary amine.

2.3. Preparation of erlotinib antibody

Five 5-week-old, female BALB/c mice (Kyudo Exp. Animals, Kumamoto, Japan) were injected intraperitoneally with 0.1 mg of EBMB-BSA conjugate emulsified in complete Freund's adjuvant. The mice received 3 injections of the con-jugate (0.05 mg) alone at 2-week intervals. Seven days after the final injection, the mice were euthanized and sera were collected, separated by centrifugation, heated at 55 °C for 30 min, and then stored at −30 °C. The anti-erlotinib serum obtained was directly used as the anti-erlotinib antibody for ELISA. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Sojo University.

2.4. Preparation of the erlotinib-HRP conjugate

Erlotinib was labeled by binding to HRP, essentially by the same method as that used for the preparation of erlotinib immunogen. EBMB (10 mg, 33.5 μmol) was dissolved in 500 μL 1 M NaOH and the solution was left to stand at 60 °C for 1 h. The resulting EBMB carboxylate was acidified by the addition of 550 μL of 1 M HCl and then extracted with ethyl acetate. The organic layer was separated and evaporated under reduced pressure. The residue was dissolved in 95% dioxane (1 mL). EDC (12.7 mg, 67 μmol) and *N*-hydroxysuccinimide (7.7 mg, 67 μmol) were added to the dioxane solution. The resulting solution was left to stand at room temperature for 2 h, and a 50 μL aliquot of the reaction mixture, containing succinimidyl EBMB, was then added directly to HRP (0.5 mg, 12.5 nmol) in 0.5 mL of 0.1 M phosphate buffer (pH 7.0), followed by incubation at room temperature for a further 2 h. The mixture was chromatographed on a column of Sephadex G-75 (2.0 cm × 30 cm) using PBS containing 0.1% BSA to remove any remaining small molecules. Fractions (4 mL)

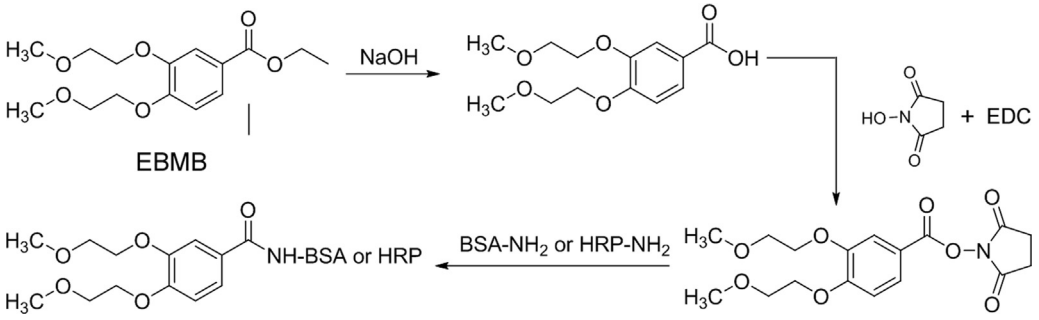

Fig. 2. Scheme showing the preparation of the immunogen and enzyme conjugate.

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