Quantification of Sorafenib in Human Serum by Competitive Enzyme-Linked Immunosorbent Assay

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The multikinase inhibitor sorafenib has been used in the treatment of hepatocellular carcinoma, renal cell carcinoma, and differentiated thyroid carcinoma. Here we have demonstrated the production of the first specific antibody against sorafenib. Anti-sorafenib serum was obtained by immunizing mice with an antigen conjugated with bovine serum albumin and carboxylic modified 4-(4-aminophenoxy)-N-methyl-2-pyridine-carboxamide (AMPC) using the N-succinimidyl ester method. Enzyme labeling of sorafenib with horseradish peroxidase was similarly performed using carboxylic modified AMPC. A simple competitive enzyme-linked immunosorbent assay (ELISA) for sorafenib was developed using the principle of direct competition between sorafenib and the enzyme marker for anti-sorafenib antibody, which had been adsorbed by the plastic surface of a microtiter plate. Serum sorafenib concentrations lower than 0.04 μg/mL were reproducibly measurable using the ELISA. This ELISA was specific to sorafenib and showed very slight cross-reactivity (2.5%) with a major metabolite, sorafenib N-oxide. The values of serum sorafenib levels from 32 patients measured by this ELISA were comparable with those measured by HPLC, and there was a strong correlation between the values determined by the two methods (r = 0.979). The specificity and sensitivity of the ELISA for sorafenib should provide a valuable new tool for use in therapeutic drug monitoring and pharmacokinetic studies of sorafenib.

Key words sorafenib; enzyme-linked immunosorbent assay; sorafenib N-oxide; multikinase inhibitor

Sorafenib, an oral multikinase inhibitor with both antiproliferative and antiangiogenic activities, is used in the treatment of hepatocellular carcinoma (HCC), renal cell carcinoma, and differentiated thyroid carcinoma.1–3 However, serious adverse effects, including liver failure, hepatic encephalopathy, and pneumonitis, arise in some cases.4 In addition, 20% of HCC patients taking sorafenib are forced to withdraw due to adverse effects within one month of starting treatment.5 Recent studies indicated that plasma cumulative exposure to sorafenib is correlated with severe adverse effects.6,7 However, significant inter-individual variability in sorafenib pharmacokinetics has been observed.8 Sorafenib is primarily metabolized by CYP3A4-mediated oxidation in the liver.9 Several studies have been performed to evaluate the potential for clinically relevant drug–drug interactions. The calcium antagonist felodipine was reported to act as an inhibitor of CYP3A4-mediated oxidation of sorafenib in a patient with HCC.10 Moreover, prednisolone, a CYP3A4 inducer, can stimulate sorafenib metabolism.11 Changes in the metabolism of sorafenib by altered CYP3A4 activity may affect its clinical and adverse effects. Evaluation of blood sorafenib levels in patients has become a useful tool for achieving the optimum therapeutic level for patients who have experienced drug interactions or adverse side effects, and for those who require dose adjustment.7,12

Several recently published studies have reported the validation of analytical methods for quantification of sorafenib in human blood using HPLC13 and liquid chromatography with tandem mass spectrometry (LC-MS/MS).14 However, these methods require expensive equipment and a high degree of technical expertise that might not be available in busy clinical chemistry departments. Thus, the development of a simple method for the quantification of sorafenib is needed. In general, the enzyme-linked immunosorbent assay (ELISA) is very sensitive and specific and has the advantages of simplicity, low cost, safety, and the ability to process numerous samples. However, there are no reports of an ELISA system for sorafenib. We previously developed several ELISAs for other tyrosine kinase inhibitors, each of which was simple, sensitive, and useful for therapeutic drug monitoring (TDM) and pharmacokinetic studies of the drugs involved.15

Here we present the first report of an ELISA protocol for sorafenib, including the methodology for antibody production, labeling of sorafenib with horseradish peroxidase (HRP) to act as a tracer, characterization of antibody specificity, and an ELISA technique developed for the measurement of sorafenib in human serum.

MATERIALS AND METHODS

Reagents Sorafenib and sorafenib N-oxide were obtained from Toronto Research Chemicals Inc. (Toronto, ON, Canada) and 4-(4-aminophenoxy)-N-methyl-2-pyridinecarboxamide (AMPC) and 2,4,6-trinitrobenzene sulfonic acid were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). HRP and 3,3’,5,5’-tetramethylbenzidine (TMB) were obtained from Boehringer Ingelheim Pharma GmbH (Ingelheim, Germany). All other reagents and solvents were of the highest grade com-

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Preparation of the Immunogen for Sorafenib  A solution of AMPC (10 mg, 41 µmol) and succinic anhydride (4.1 mg, 41 µmol) in pyridine (0.5 mL) was stirred overnight at 60°C. After the addition of water, the resulting mixture was extracted with ethylacetate. The ethylacetate solution was dried over anhydrous sodium sulfate. The solvent was removed by evaporation, and the residue, carboxylic modified AMPC, was dissolved in 95% dioxane (0.5 mL). The dioxane solution was added to a 1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride (EDPC) (12.5 mg, 65 µmol) and N-hydroxysuccinimide (7.5 mg, 65 µmol), and the resulting solution was allowed to stand at room temperature for 2 h. The reaction mixture containing succinimidy AMPC was immediately mixed with bovine serum albumin (BSA) (20 mg) in 1 mL of 0.1 M phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% β-cyclodextrin, and 0.1% BSA (buffer A) to remove any remaining small molecules from the mixture. Fractions (3 mL) were collected and fractions 12 to 14, representing the main peaks showing enzyme activity, were combined and used as a label in the ELISA.

Preparation of the Sorafenib–HRP Conjugate  Sorafenib was labeled by binding to HRP, essentially by the same method used for the preparation of the sorafenib immunogen. A solution of AMPC (10 mg, 41 µmol) and succinic anhydride (4.1 mg, 41 µmol) in pyridine (0.5 mL) was stirred overnight at 60°C. After the addition of water, the resulting mixture was extracted with ethylacetate. The ethylacetate solution was dried over anhydrous sodium sulfate. The solvent was removed by evaporation, and the residue, carboxylic modified AMPC, was dissolved in 95% dioxane (0.5 mL). The dioxane solution was added to EDPC (12.5 mg, 65 µmol) and N-hydroxysuccinimide (7.5 mg, 65 µmol), and the resulting solution was allowed to stand at room temperature for 2 h. A 30 µL aliquot of the reaction mixture containing succinimidy AMPC was added directly to HRP (1 mg, 25 nmol) in 1 mL of 0.1 M phosphate buffer (pH 7.0), followed by 1 h of incubation at room temperature. Chromatography was carried out on a column of Sephadex G-75 (2.0×30 cm) using 20 mM phosphate buffer (pH 7.0) containing 3 M urea. The purified conjugate was used as the immunogen for ELISA. Using the trinitrobenzene sulfonic acid method to determine the primary amine, about 20 AMPC molecules were coupled with each molecule of BSA, based on the decrease in the primary amine.

Antibody Generation  Six 5-week-old, female BALB/c mice (Kyudo Exp. Animals; Kumamoto, Japan) were injected intraperitoneally (i.p.) with 0.1 mg of sorafenib–BSA conjugate emulsified in Complete Freund’s adjuvant (Difco, Tokyo, Japan). Subsequently, three injections of 0.05 mg of the conjugate were given alone at two-week intervals. Seven days later, the mice were killed and sera were separated by centrifugation, heated at 55°C for 30 min, and stored at −30°C. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Sojo University.

Serum Samples  This study was approved by Shiga Committee for Animal Experimentation of Sojo University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Sojo University.

Sample Preparation  An aliquot of serum sample (10 µL) was subjected to protein precipitation with dimethyl sulfoxide (DMSO) (20 µL) and carefully vortex-mixed. The mixture was centrifuged at 10480×g for 5 min at room temperature. A 15 µL aliquot of the supernatant was added to 485 µL of buffer A, and the mixture was analyzed directly by ELISA. However, when the sorafenib concentration of serum sample was 5 µg/mL or more, the serum sample diluted 10-fold with normal human serum to obtain sorafenib concentration appropriate for a measurement by ELISA.

ELISA Procedure  ELISA is based on the principle of competition between enzyme-labeled and unlabeled drugs for an immobilized antibody, followed by measurement of the marker enzyme activity of the immunocomplex bound to the solid phase. Briefly, wells in microtiter plates (Nunc F Immunoplates I; Nunc, Reskilde, Denmark) were coated by loading 100 µL of anti-sorafenib serum diluted 1:5000 in 10 mM Tris–HCl buffer (pH 8.5) containing 10 mM sodium chloride (NaCl) and 10 mM NaN3 and allowed to stand for 1 h at 37°C. After the plates had been washed twice with buffer A, they were incubated with 100 µL of 10 mM Tris–HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM NaN3 with 1% skim milk for 30 min at 37°C to prevent nonspecific adsorption. The anti-sorafenib antibody-coated wells were filled with 50 µL of either sorafenib-treated samples or buffer A as a control, followed immediately by 50 µL of the pooled sorafenib–HRP conjugate (diluted 1:100 in buffer A for sorafenib). The wells were then incubated overnight at 4°C and again washed thoroughly with buffer A.

The activity of the enzyme conjugate bound to each well was measured by the addition of 100 µL of 0.42 mM TMB in 0.05 M acetate–citric acid buffer (pH 5.5) containing 3% DMSO and 0.01% hydrogen peroxide, followed by incubation of the wells at 37°C for a suitable period. The enzyme reaction was stopped by the addition of 100 µL of 2.0 mM sulfuric acid (H2SO4) to each well, and the resulting color intensity was measured spectrophotometrically at 450 nm using an ELISA analyzer (ImmunoMini NJ-2300, Nalje Nunc Int. Co., Ltd., Tokyo, Japan). Concentrations were calculated from the standard curve using semi-logarithmic graph paper.

Fig. 1. Chemical Structures of Sorafenib and Its Major Metabolite  
Sorafenib  
Sorafenib N-oxide
University of Medical Science Hospital Ethics Committee (22-32-2). After obtaining written informed consent from the patients, serum samples were collected in sterilized vacuum tubes for serum separation. The samples were centrifuged (2610 × g at 4°C for 10 min), and the harvested serum was stored at −30°C until analysis.

**HPLC Method** The HPLC method for sorafenib in human serum was performed according to a previous method.11

**RESULTS AND DISCUSSION**

Sorafenib N-oxide, which is formed by CYP3A4-mediated metabolism, is the main circulating metabolite in human plasma and has similar in vitro potency to sorafenib9 (Fig. 1). In several clinical trials, sorafenib accounted for approximately 70–85% of circulating analytes in plasma, and sorafenib N-oxide accounted for approximately 9–17%.9) Therefore, to develop an ELISA that can specifically measure the concentration of sorafenib in human plasma, an anti-sorafenib antibody that does not show cross-reaction with sorafenib-N-oxide must be produced. In general, the antibody specificity on the hapten appears to be towards the group farthest away from the region of conjugation to the carrier protein in the immunogen structure.17,18) Thus, to produce an antibody specific for the N-methyl-2-pyridinecarboxamide moieties of sorafenib, the immunogen was prepared using part of the structure of sorafenib (Fig. 2).

Carboxylic modified AMPC was coupled to BSA using the hydroxysuccinimide ester method,19) and the resulting AMPC–BSA conjugate (sorafenib immunogen), with about 20 mol of AMPC per mol of BSA, induced the formation of specific antibodies in each of the five mice immunized. The anti-sorafenib serum obtained was directly adsorbed to the wells in the microtiter plates.

AMPC–HRP conjugate (as a tracer) was prepared by the same procedure. The conjugate obtained was stable for over 6 months in eluted buffer (pH 7.0) at 4°C without any loss of the enzyme or its immunoreactivity.

The dose–response standard curves of sorafenib obtained in

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**Fig. 2.** Scheme Showing the Preparation of the Immunogen and Enzyme Conjugate in ELISA for Sorafenib

**Fig. 3.** Standard Curve for Sorafenib in Buffer and Human Serum

The curve shows the ratio (%) of bound enzyme activity for various doses of sorafenib (B) to that using sorafenib–HRP alone (B₀). Buffer: ◦, human serum: ■

**Fig. 4.** Standard Curve for Sorafenib in Deproteinized Human Serum

The curve shows the ratio (%) of bound enzyme activity for various doses of sorafenib (B) to that using sorafenib–HRP alone (B₀). The curve was essentially linear on a semilogarithmic plot between 0.008 and 25 µg/mL. Each point represents the mean ± S.D. (n=3).
the buffer and human serum are shown in Fig. 3. The range of sorafenib detection by ELISA in the buffer and human serum were 0.00016 to 0.5 µg/mL and 0.004 to 12.5 µg/mL, respectively. The quantity of sorafenib was substantially decreased by adding normal human serum (50 µL). This observation suggested the presence of a serum protein(s), which interfered with the ELISA. It is generally well-known that the serum protein level fluctuates by various pathological condition. Therefore, serum samples were deproteinized by DMSO to exclude interference by serum proteins. The dose–response standard curve of sorafenib obtained in the deproteinized human serum is shown in Fig. 4. The curve was essentially linear on a semilogarithmic plot between 0.008 and 25 µg/mL.

For practical purposes, the working range was arbitrarily set between 0.04 and 5 µg/mL based on the precision and accuracy findings for the ELISA in serum (Table 1), which showed that our technique is reproducible. Recoveries of four different sorafenib levels ranging from 0.04 to 5 µg/mL were satisfactory (94.5–102.8%, n=5). The coefficients of variation for intra-and inter-assays at four different sorafenib levels between 0.04 to 5 µg/mL were 5.0–9.5% and 4.9–9.7% (n=5 in all cases), respectively. The detection limit of sorafenib in the ELISA was 0.04 µg/mL (Student’s t-test, n=3, p<0.01 compared with the B0 value). The plasma concentration range at clinical doses of sorafenib is between about 2 and 9 µg/mL. Therefore, this ELISA may be sufficiently sensitive to quantify sorafenib in TDM and pharmacokinetic studies.

Antibody specificity was determined by measuring the displacement of bound sorafenib–HRP by AMPC used as a hapten antigen, sorafenib N-oxide (the major metabolite), and compounds of a similar structure. Values of cross-reactivity were defined as the ratio of each compound to sorafenib in the concentrations required for 50% inhibition of sorafenib–HRP binding to the antibody. The anti-sorafenib antibody showed 100.0% cross-reactivity with AMPC, 83.3% with regorafenib, 2.5% with sorafenib N-oxide, and 0.44% with enzalutamide. No detectable cross-reaction, however, was found with 4-phenoxypyridine (Table 2). These findings suggest that the antibody recognizes almost the whole structure of AMPC satisfactorily, and is sufficiently specific to the structure of sorafenib. During long-term therapy, the plasma level of the

Table 1. Precision and Recovery of ELISA for Sorafenib in Human Serum

| Added (µg/mL) | Estimated (µg/mL) | Recovery (%) | CV (%) |
|--------------|------------------|--------------|--------|
| Intra-assay  |                  |              |        |
| 0.04         | 0.0378±0.003     | 94.5         | 7.9    |
| 0.2          | 0.197±0.016      | 98.5         | 8.1    |
| 1.0          | 1.028±0.0511     | 102.8        | 5.0    |
| 5.0          | 5.14±0.4883      | 102.8        | 9.5    |
| Inter-assay  |                  |              |        |
| 0.04         | 0.0396±0.0024    | 99.0         | 6.1    |
| 0.2          | 0.1904±0.0107    | 95.2         | 5.6    |
| 1.0          | 1.0216±0.0988    | 102.2        | 9.7    |
| 5.0          | 4.988±0.2445     | 99.8         | 4.9    |

Values represent the mean±S.D. of 5 experiments.

Table 2. Specificity of Anti-sorafenib Antibody

| Compounds                  | % Cross-reaction (50%) |
|----------------------------|------------------------|
| Sorafenib                  | 100.0                  |
| AMPC                       | 100.0                  |
| Regorafenib                | 83.3                   |
| Sorafenib N-oxide          | 2.5                    |
| Enzalutamide               | 0.44                   |
| 4-Phenoxypyridine          | <0.01                  |
Determined by the Present ELISA and HPLC Methods

Fig. 5. Correlation between Serum Sorafenib Concentrations of Patients Determined by the Present ELISA and HPLC Methods

major metabolite, sorafenib N-oxide, is about 9–17% of the plasma level of sorafenib. Therefore, the major metabolite likely did not cause interference, considering the specificity of the antibody and the plasma level of the major metabolite. On the other hand, sorafenib also undergoes glucuronidation by the uridine diphosphate glucronosyl transferase (UGT1A9) pathway. Approximately 19% of the administrated sorafenib dose is excreted in urine, almost exclusively as glucuronol conjugates of the parent drug and metabolites, but not unchanged sorafenib. The cross-reactivity of these glucuronide conjugates of the parent drug and metabolites has not yet been confirmed. However, it is estimated that the glucuronide metabolite of parent drug shows a similar cross-reaction to sorafenib judging from the specificity of the anti-sorafenib antibody.

The ELISA method was compared with HPLC in the measurement of serum samples from 32 patients (Fig. 5). Good correlation between the values of the two methods was found. The equation $Y=1.016X-0.137$ was derived, where $Y$ was the concentration determined by HPLC analysis and $X$ was that determined by ELISA, and a correlation coefficient of 0.979 ($n=32$) was calculated. These results suggest that this ELISA may be specific enough to quantify sorafenib for TDM and pharmacokinetic studies in humans.

In summary, the ELISA procedure for sorafenib reported here is sensitive, specific, reproducible, simple, and adaptable for use in the analyses of numerous samples. This ELISA will be a valuable tool in TDM and in pharmacokinetic studies of sorafenib.

Conflict of Interest The authors declare no conflict of interest.

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