Development of a Specific and Sensitive Enzyme-Linked Immunosorbent Assay for the Quantification of Imatinib

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Imatinib is an oral tyrosine kinase inhibitor used for first-line treatment of chronic myeloid leukemia. Therapeutic drug monitoring targeting trough plasma levels of about 1000 ng/mL may help to optimize imatinib’s therapeutic effect. This paper reports a specific and sensitive enzyme-linked immunosorbent assay (ELISA) for a pharmacokinetic evaluation of imatinib. Anti-imatinib antibody was obtained by immunizing rabbits with an antigen conjugated with bovine serum albumin and succinimidyl 4-[(4-methyl-1-piperazinyl)methyl]-benzoate. Enzyme labeling of imatinib with horseradish peroxidase was similarly performed using succinimidyl 4-[[4-methyl-1-piperazinyl]methyl]-benzoate. A simple ELISA for imatinib was developed using the principle of direct competition between imatinib and the enzyme marker for anti-imatinib antibody which had been adsorbed by the plastic surface of a microtiter plate. Serum imatinib concentrations lower than 40 pg/mL were reproducibly measurable using the ELISA. This ELISA was specific to imatinib and showed very slight cross-reactivity (1.2%) with a major metabolite, N-desmethyl imatinib. Using this assay, drug levels were easily measured in the blood of mice after their oral administration of imatinib at a single dose of 50 mg/kg. The specificity and sensitivity of the ELISA for imatinib should provide a valuable new tool for use in therapeutic drug monitoring and pharmacokinetic studies of imatinib.

Key words imatinib; enzyme-linked immunosorbent assay; N-desmethyl imatinib

MATERIALS AND METHODS

Reagents Imatinib mesylate was obtained from LKT Labs.

The authors declare no conflict of interest.

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oratories, Inc. (St. Paul, MN, U.S.A.). N-Desmethyl imatinib was obtained from TLC Pharmachem., Inc. (Ontario, Canada). 4-[(4-Methyl-1-piperazinyl)methyl]-benzoic acid (MPMB) dihydrochloride and 2,4,6-trinitrobenzene sulfonic acid were 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). All other reagents and solvents were of the highest grade commercially available.

**Preparation of the Immunogen for Imatinib** 1-Ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride (EDPC) (12.5 mg, 65 µmol) and N-hydroxysuccinimide (7.5 mg, 65 µmol) were added to a solution of MPMB dihydrochloride (10 mg, 32.5 µmol) in 95% dioxane (2 mL), and the resulting solution was allowed to stand at room temperature for 2 h. The reaction mixture containing succinimidyl MPMB was immediately mixed with bovine serum albumin (BSA) (20 mg) in 4 mL of 0.1 M phosphate buffer (pH 7.0) and incubated at room temperature for 2 h. The reaction mixture was dialyzed successively for 96 h against 10 mM Tris–HCl buffer (pH 8.5) containing 10 mM 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 150 µL of either imatinib-treated samples or buffer A as a control, followed immediately by 50 µL of the pooled imatinib-HRP conjugate (diluted 1:100 in buffer A for imatinib). The wells were then incubated overnight at 4°C and once again washed thoroughly with buffer A.

The activity of the enzyme conjugate bound to each well was then measured by the addition of 150 µL of 0.42 mM TMB in 0.05 M4 acetate–citric acid buffer (pH 5.5) containing 3% dimethyl sulfoxide 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 150 µL of 1 M 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.

**Pharmacokinetic Evaluation** Three female BALB/c mice (Kyudo Exp. Animals; Kumamoto, Japan) with a weight range of 25–31 g were used in this study. Imatinib was orally administered at a dose of 50 mg/kg to the mice using a sonde after an overnight fast. The drug was suspended in a carrier composed of isotonic NaCl at a concentration of 5 mg/mL. Peripheral blood (5 µL) was collected from the tail vein pre-administration and at 0.25, 0.5, 1, 1.5, 2, 4, 6, and 8 h after administration; diluted 200 times with PBS; and immediately vortexed for several seconds. Diluted blood samples were centrifuged at 3000×g for 10 min at 4°C, and each resulting supernatant was stored at −30°C until it could be assayed for imatinib concentration. Blood samples were diluted 10-fold with buffer A to obtain imatinib concentrations appropriate for measurement by ELISA. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Sojo University.

**RESULTS AND DISCUSSION**

To attain antigenicity, imatinib must be conjugated with proteins, but it lacks the free amino and carboxyl groups which are generally used as conjugation sites. To develop an ELISA for imatinib which can be applied to TDM and pharmacokinetic study, it is necessary to produce an anti-imatinib antibody that does not cross-react with imatinib’s major metabolite, N-desmethyl imatinib. In general, the antibody specificity of hapten appears to be toward the group farthest away from the region of conjugation of the carrier protein in the immunogen structure. Therefore, to produce an antibody specific to the methyl piperazin moiety of imatinib, imatinib immunogen was prepared from a partial structure of imatinib itself (MPMB) (Fig. 2). The MPMB was coupled to BSA...
using the hydroxysuccinimide ester method, and the resulting MPMB-BSA conjugate (imatinib immunogen), with about 20 mol of MPMB per mol of BSA, induced the formation of specific antibodies in each of the two rabbits immunized. MPMB-HRP conjugate (as a tracer) was also prepared by the same procedure. The conjugate obtained was stable for more than 6 months in eluted buffer (pH 7.0) at 4°C without any loss of the enzyme or immunoreactive enzyme activity.

Using the anti-imatinib antibody and imatinib-HRP as a tracer, an ELISA for the quantification of imatinib was developed. The dose–response standard curve of imatinib obtained in the human serum is shown in Fig. 3. The curve was essentially linear on a semilogarithmic plot between 8 pg and 25 ng/mL. For practicality, the working range was arbitrarily set between 40 and 5000 pg/mL based on the precision and accuracy findings for the ELISA in serum (Table 1), which showed our technique to be reproducible. Recoveries of four different imatinib levels ranging from 40 to 5000 pg/mL were satisfactory (98.0% to 104%, n=5). The coefficients of variation for intra- and interassays at four different imatinib levels between 40 to 5000 pg/mL were 4.5% to 14.7% and 4.1% to 11.6% (n=5 for all cases), respectively. The detection limit of imatinib in the ELISA was 40 pg/mL (Student’s t-test, n=3, p<0.01 compared with the B₀ value). This sensitivity appears to be about 250 times greater than the previously reported LC-MS/MS. In patients with CML, the steady-state trough plasma imatinib levels must exceed 1002 ng/mL to obtain clinical benefits, and these plasma concentrations of imatinib were associated with a clinical response. Therefore this ELISA may be sufficiently sensitive to quantify imatinib in TDM and pharmacokinetic studies.

Antibody specificity was determined based on the displacement of bound imatinib-HRP by similar compounds. Values of cross-reactivity were defined as the ratio of each compound to imatinib in the concentrations required for 50% inhibition of imatinib-HRP binding to the antibody. The anti-imatinib antibody showed 100.0% cross-reactivity with MPMB used as a hapten antigen, 1.2% with N-desmethyl imatinib (the major metabolite), 0.15% with 4-piperazin-1-ylmethyl-benzoic acid methylester, and 0.01% with 1-methylpiperazine (Table 2). These findings suggest that the antibody well recognizes almost the whole structure of the MPMB, and thus is sufficiently specific to the structure of imatinib.

Figure 4 shows the time course of the blood concentrations of imatinib following its oral administration at a dose of 50 mg/kg to the mice. Imatinib was rapidly absorbed, reached a peak concentration in the blood of 6.75±0.61 µg/mL (mean±S.D.) at 60 min after dosing, and then slowly decreased. The imatinib levels in the blood tested were almost consistent with those reported by Tan et al. by means of HPLC for imatinib. Using this ELISA, imatinib levels were easily measured in the blood of mice after oral administration. The ELISA determinations, however, may have measured the total amount of imatinib immunoreactivity including imatinib metabolites with cross-reactivity with anti-imatinib antibodies, such as
the major metabolite of imatinib in humans, N-desmethyl imatinib. However, during a long-term therapy, the plasma concentration of N-desmethyl imatinib was found to be only about 17% of the plasma concentration of imatinib. Therefore, considering the specificity of antibody and plasma concentrations of N-desmethyl imatinib, there may have been no interference with imatinib. The cross-reactivity of the other metabolites has not yet been confirmed; however, the maximal concentration of these metabolites detected in human plasma was relatively low. Therefore, this ELISA may be sufficiently specific to quantify imatinib for TDM and pharmacokinetic studies in humans.

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

Fig. 4. Blood Imatinib Levels in Mice after a Single Oral Administration of Imatinib

Three mice with a weight range of 25–31 g were injected with 50 mg/kg imatinib. At each interval, blood was collected and blood imatinib level was measured by ELISA. Each point represents the mean±S.D. (n=3).

Table 2. Specificity of Anti-imatinib Antibody

| Compounds                        | Cross-reactivity (%) |
|----------------------------------|----------------------|
| Imatinib                         | 100.0                |
| 3-(4-Methylpiperazin-1-ylmethyl)benzoic acid | 100.0                |
| N-Desmethyl imatinib             | 1.2                  |
| 4-Piperazin-1-ylmethyl-benzoic acid methylester | 0.15                 |
| 1-Methylpiperazine               | 0.01                 |

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