Enzyme Immunoassay for the Drug of Anti-ulcer Using Avidin–Biotin System

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We have developed a competitive enzyme immunoassay for a drug, which was a newly synthesized anti-ulcer agent, using an enzyme immunoassay. The polyclonal anti-drug antibody coupled to biotin, peroxidase labeled drug derivatives as a tracer, and a small column of Sepharose 4B covalently bound to avidin were used in the assay. This assay is simple and rapid, and the sensitivity and the measuring range can be controlled by the flow rate of the substrate solution. The correlation between serum drug concentrations (0.1–10 ng/ml) measured by gas chromatography and this assay was good ($r = 0.991$). This principle for the assay is very practical and applicable to the enzyme immunoassay for small and large molecules.

Keywords enzyme immunoassay; anti-ulcer agent; avidin; biotin; peroxidase

The determination of drugs in body fluids must be specific and rapid in order to obtain the clinical data of the patients. 11 Therapeutic drug monitoring is necessary to depress the adverse reaction of the drug in the case of the narrow therapeutic range between its efficacy and toxicity. Effective treatment is furthermore complicated because the pharmacokinetics of therapeutic drugs are significantly changed in individual patients. 21 Measurement of therapeutic drugs is now provided as a service by the department of clinical chemistry or pharmacy. 3 Several techniques have been used for measuring therapeutic drugs, including ultraviolet spectroscopy, 4 gas chromatography, 5 high performance liquid chromatography 6 and enzyme immunoassay. 7

Since newly synthesized $(−)-2,3,3$-dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzoiazepin-4(SH)-one hydrochloride (BTM-1086) was a strong potential anti-ulcer agent, we have attempted to develop a determination system to evaluate the anti-ulcer activity. In a previous paper, 3 we had prepared the rabbit anti-BTM-1086 antibody and developed the competitive enzyme immunoassay which is not ideally suited as a drug monitoring method since it involves a lot of work and is time-consuming.

In this paper, we describe a simple and rapid assay system for BTM-1086 using the avidin-biotin binding reaction.

Materials and Methods

Materials BTM-1086 and its metabolites were synthesized by Maruko Seiyaku Co., Ltd., as shown in Fig. 1. Horse radish peroxidase (HRP type VI) was obtained from Sigma Chemical Co. (U.S.A.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and glutaraldehyde were from Nakarai Chemicals, Ltd. (Kyoto). Bovine serum albumin (BSA fraction V) was from Wako Pure Chemical Industries, Ltd. (Osaka). All other solvents and chemicals were of special grade. Rabbit anti-BTM-1086 antibody and BTM-1086-HRP conjugate were prepared according to the method of the previous paper. 10

Buffer A 50 nm sodium phosphate buffer, pH 7.4, containing 0.02% Triton X-405 and 0.3 m NaCl.

Preparation of Biotinyl Antibody Six ml of anti-BTM antibody purified by protein A-Sepharose 4B was dissolved in 10 nm sodium phosphate buffer (pH 7.4) containing 0.15 m NaCl (2 mg protein/ml), then mixed with 200 ml of biotinyl N-succinimide dissolved in dimethylformamide (2 mg/ml). The mixture was allowed to stand for 2 h at room temperature according to the method of Jasieiwicz, et al. 9 The resulting mixture was dialyzed against phosphate buffered saline at 4°C overnight and diluted with buffer A before use.

Coupling of Avidin to Sepharose 4B Avidin was covalently linked to Sepharose 4B (10 mg avidin/30 ml gel) according to the method of March et al. 10

Standard Procedure After 30 ml of normal rat serum as a carrier, 10 ml of serum sample and 40 ml of buffer A were mixed, 40 ml of BTM-1086-HRP conjugate (diluted 1:200 with buffer A) and 10 ml of biotinyl antibody solution (diluted 1:20 with buffer A) were added and the mixture was allowed to stand at room temperature for 20 min. 60 ml of the mixture was applied to column (4.4 × 13 mm) of 0.2 ml avidin coupled to Sepharose 4B, and the column was washed with 4 ml of buffer A at a flow rate of 7.5 ml/min. 0.5% of 3-(p-hydroxyphenyl)propionic acid solution, buffer A and 0.005% hydrogen peroxide solution were mixed at the ratio of 1:10:1 and used as a substrate solution. The substrate solution was applied on to the column at the same flow rate and after 5 min the fraction 0.5 ml was collected. 1.6 ml of 0.1 N NaOH were added into the 0.4 ml out of the 0.5 ml and the fluorescence of the resulting solution was measured at 320 nm for excitation and 405 nm for emission with a Hitachi model FPL-2 fluorometer. The calibration curve was prepared using the standard BTM dissolved in normal serum.

Correlation between Enzyme Immunoassay (EIA) and Gas Chromatography (GC) Wistar male rats were bled from the descending aorta at 0.5, 1, 2, 4, 6, 9 and 24 h after a single oral administration of BTM-1086 (30 mg/kg). The serum samples were assayed for BTM-1086 by EIA and GC. GC was performed on a Hitachi 163 gas chromatograph equipped with a hydrogen flame thermionic detector, and the conditions used were as follows; 1 m × 3 mm glass column packed with 2% OV-17 Chromosorb W (AW, DMCS); carrier gas, He, 50 ml/min; injection port temperature, 300°C; column temperature, 280°C.

Results and Discussion

Antigen–Antibody Reaction According to the standard assay system, we investigated the antigen–antibody reaction. The time course of the antigen–antibody reaction is shown in Fig. 2. The rate of the reaction was found to reach plateau
within 15 min. The antigen–antibody reaction was known to be accelerated by the addition of polyethyleneglycol (0.1—0.05 M). In this experiment, we examined the influence of polyethyleneglycol on the reaction, however, there is no difference between the rate of reactions in the presence and the absence of polyethyleneglycol.

**Standard Curve for BTM-1086** The standard curve for BTM-1086 is obtained according to the standard assay as shown in Fig. 3. The measuring range was 0.05—20 μg/ml corresponding to the 0.5—200 ng/tube.

**TABLE I. The Influence of Flow Rate of Substrate Solution on the Sensitivity of the Assay System**

<table>
<thead>
<tr>
<th>Flow rate (ml/h)</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BTM-1086 (0 μg/ml)</td>
</tr>
<tr>
<td>3.8</td>
<td>313.6</td>
</tr>
<tr>
<td>7.5</td>
<td>200.6</td>
</tr>
<tr>
<td>15.0</td>
<td>112.9</td>
</tr>
<tr>
<td>30.0</td>
<td>42.3</td>
</tr>
</tbody>
</table>

**TABLE II. Specificity of Antiserum against BTM-1086**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTM-1086</td>
<td>100.0</td>
</tr>
<tr>
<td>BTM-1066</td>
<td>11.5</td>
</tr>
<tr>
<td>BTM-1091</td>
<td>0.9</td>
</tr>
<tr>
<td>BTM-1092</td>
<td>18.9</td>
</tr>
<tr>
<td>BMS-053</td>
<td>0.4</td>
</tr>
<tr>
<td>Desmethyl-BMS-053</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The sensitivity of the assay depends on the flow rate of the substrate solution as shown in Table I. The slower flow rate offers more high sensitivity. In this assay system, 7.5 ml/h of flow rate is adopted.

**Recovery of BTM-1086 from Sera** We assayed the analytical recovery by using a human pool serum supplemented with different concentrations of the standards. Mean recoveries ranged from 98.5 to 101.4% for all the assays.

**Specificity** The results of the cross reaction study are summarized in Table II. The cross-reaction percentage was calculated at 50% displacement of the BTM-1086–HRP conjugate in the EIA system. Cross reactivity with BTM-1086 metabolites, BMS-053 and desmethyl BMS-053 is below 1% in this assay system. The cross reactivity with BTM-1086 antibody was almost identical with the results in the previous paper.

**Accuracy and Precision** The inter and intra-assay coefficient of variation (C.V.) of serum samples were 3.95 and 4.07% at a level of 4 μg/ml, respectively.

**Correlation between EIA and GC** The serum levels of BTM-1086 were determined by the proposed method and gas chromatography. There was a good correlation ($r = 0.991$) between two assays as shown in Fig. 4.
Serum Level of BTM-1086 after Oral or Intravenous Administration The serum levels of BTM-1086 in rat are shown in Fig. 5. In the case of a single intravenous injection (5 mg/kg), the pharmacokinetic parameters are almost the same as those in the previous paper. It was found that β-phase in the excretion after p.o. administration of BTM-1086 is identical to that in the case of i.v. administration.

Although the sensitivity of the enzyme immunoassay used in this paper is lower than the previous immunoassay, this assay system is more simple and rapid. Serum levels of BTM-1086 determined by EIA correlated well with those of GC. This suggests that the lower sensitivity is not a practical problem in the determination of the serum level of BTM-1086. These results indicated that this enzyme immunoassay of the drug is very convenient for monitoring the serum level of an active drug.

References