Determination of Topotecan by ELISA

Tetsuya Saita,* Hiroshi Fujito, and Masato Mori
Faculty of Hospital Pharmacy, Saga Medical School, 5–1–1 Nabeshima, Saga 849–8501, Japan.
Received November 24, 2000; accepted January 10, 2001

* To whom correspondence should be addressed. e-mail: saita@post.saga-med.ac.jp © 2001 Pharmaceutical Society of Japan

A highly sensitive ELISA for the determination of (s)-9-dimethylaminomethyl-10-hydroxy-camptothecin (topotecan) capable of measuring as low as 80 pg/ml was developed. Anti-topotecan antibody was obtained by immunizing rabbits with topotecan conjugated with bovine serum albumin using diazotized m-aminobenzoic acid as a cross-linker. Enzyme labeling of topotecan with β-D-galactosidase was performed by utilizing another cross-linker, N-(4-diazophenyl)maleimide. The specificity of this ELISA appears to be primarily toward the lactone moiety of topotecan, showing a very slight cross-reactivity with the lactone opened-ring of topotecan. The values for the topotecan concentrations detected using this assay were comparable with those detected using HPLC. There was a good correlation between the values determined by the two methods. These findings suggest that this ELISA can detect the natural amounts of the lactone form. Using this assay, drug levels were easily determined in the blood and urine of rats for 5 h after i.v. administration of topotecan at a single dose of 1 mg/kg. The sensitivity and specificity of the ELISA should provide a useful tool for developing pharmacokinetic and pharmacodynamic studies of topotecan.

Key words topotecan; ELISA; anti-cancer drug

Topotecan ([s]-9-dimethylaminomethyl-10-hydroxy-camptothecin) is a semisynthetic water-soluble analog of the plant alkaloid camptothecin and has exhibited antitumor activity in experimental tumor models.1,2) The mechanism of action of alkaloid camptothecin and has exhibited antitumor activity in tothecin) is a semisynthetic water-soluble analog of the plant (Fig. 1).6) The lactone form is rapidly and spontaneously con-

Preparation of the Immunogen for Topotecan The re-
actions involved in the preparative process used in the pre-
sent study are shown in Fig. 2. m-Aminobenzoic acid (m-
ABA) (2 mg, 14.5 m mol) in 200 μl 2 N hydrochloric acid was diazotized with sodium nitrite (2 mg, 29 m mol) in 50 μl distilled water at 0°C for 10 min. Next, 190 μl portions of the above reaction mixture containing diazotized m-ABA (ca. 11 μmol) was added directly to topotecan (5 mg, 11 μmol) in 10 ml of 0.5 M phosphate buffer (pH 7.5), followed by incubation at room temperature for 30 min with vigorous stirring. The solvent was mixed with 10 ml each of ethyl acetate and 0.33 M citric acid. The mixture was shaken vigorously, and the ethyl acetate layer containing topotecan–m-ABA was separated. The ethyl acetate layer was washed with saturated sodium chloride and drying over anhydrous sodium sulfate. The resulting topotecan–m-ABA was used without further purification for preparation of the conjugates using bovine serum albumin (BSA) as the topotecan immunogen. The yield of topotecan–m-ABA was tentatively estimated to be 52.0% according to HPLC measurements of the quantity of non-reacted topotecan. The molar extinction coefficients of topotecan–m-ABA were thus estimated to be 8200 at 280 nm.

Materials and Methods

Reagents Topotecan, as a hydrochloride salt (SKF 104864-A; lot. MM-19163-58), was supplied by Smithkline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). 7-Ethyl-10-hydroxycamptothecin (SN-38) was supplied by Yakult Honsha Co., Ltd. (Tokyo, Japan). Camptothecin was purchased from Aldrich Chem. Co. (St. Louis, MO, U.S.A.). 2-Hydroxymethyl-3-[1-(N-isopropylcarbamoyl)-1-hydroxypropyl]indolizino[1,2-b]quinoline-1-(11-H)-one (campto-
thecin-21-isopropylamide) was synthesized by the method of Adamovics et al.9) β-D-Galactosidase (β-D-Gal; EC 3.2.1.23) from Escherichia coli and 4-methylumbelliferyl-β-D-galactopyranoside were obtained from Boehringer Mannheim (Mannheim, Germany). N-(4-Aminophenyl)maleimide (APM) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

We succeeded in developing the first specific and sensitive ELISA for topotecan. The present study reports the method-
ology for the antibody production, the labeling of topotecan with β-D-galactosidase (β-D-Gal) to act as a tracer, the charac-
terization of antibody specificity, and the technique devel-
oped for the measurement of topotecan by ELISA. The initial appli-
cation of the assay to the measurement of topotecan lev-
els in rats demonstrates its usefulness for the assessment of basic pharmacokinetic distributions.

Fig. 1. Equilibrium Reactions between Topotecan and Its Carboxylate
and 16100 at 380 nm, representing those needed to evaluate the quantity of topotecan conjugated per mole of BSA.

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDPC) (2.3 mg, 12 \( \mu \)mol) and \( N \)-hydroxysuccinimide (1.4 mg, 12 \( \mu \)mol) were added to a solution of topotecan–1-ABA (approximately 6 \( \mu \)mol) in 1 ml of 95% dioxane, and the resulting solution was allowed to stand at room temperature for 2 h. The reaction mixture was extracted with ethyl acetate, washed with water, dried over anhydrous sodium sulfate, and evaporated. The residue was mixed with 0.5 M phosphate buffer (pH 7.5), followed by incubation at room temperature for 15 min with vigorous stirring. The solvent was mixed with 2 ml each of ethyl acetate and 0.33M citric acid. The mixture was shaken vigorously, and the ethyl acetate layer containing topotecan–DPM was separated. Then, the ethyl acetate layer was washed with saturated sodium chloride and dried over anhydrous sodium sulfate. The resulting topotecan–DPM was used without further purification for the coupling reaction with BSA.

Preparation of the Topotecan–β-Gal Conjugate

Topotecan was labeled by binding to β-Gal, essentially by the same principle used for the previous preparation of irinotecan immunogen, using a cross-linker \( N \)-(4-diazophenyl) maleimide (DPM).\(^{11}\) APM (1 mg, 5.3 \( \mu \)mol) in 20 \( \mu \)l DMF was acidified by the addition of 50 \( \mu \)l of 1 \( \mu \)l acetic acid and then diazotized with sodium nitrite (0.75 mg, 10.9 \( \mu \)mol) in 50 \( \mu \)l distilled water at 0 \( ^\circ \)C for 10 min. Next, 50 \( \mu \)l portions of the above reaction mixture containing DPM (ca. 2.2 \( \mu \)mol) was added directly to topotecan (1 mg, 2.2 \( \mu \)mol) in a mixed solution comprising 100 \( \mu \)l of DMF and 1 ml of 0.5 M phosphate buffer (pH 7.5), followed by incubation at room temperature for 15 min with vigorous stirring. The solution was mixed with 2 ml each of ethyl acetate and 0.33 M citric acid. The mixture was shaken vigorously, and the ethyl acetate layer containing topotecan–DPM was separated. Then, the ethyl acetate layer was washed with saturated sodium chloride and dried over anhydrous sodium sulfate. The resulting topotecan–DPM was used without further purification for the coupling reaction with β-Gal. The yield of topotecan–DPM was tentatively estimated to be 32% according to HPLC measurements of the quantity of non-reacted topotecan. Fifty microliters of DMF solution containing topotecan–DPM (approximately 35 \( \mu \)g, 56 nmol) was mixed with β-Gal (156 \( \mu \)g, 0.28 nmol) in 1 ml of 0.1 M phosphate buffer (pH 6.0), followed by 30 min incubation at room temperature. The mixture was chromatographed on a Sepharose 6B column (2.0 \( \times \)35 cm) using 20 mM phosphate buffer (pH 6.0) containing 0.1 M NaCl, 1 mM MgCl\(_2\), 0.1% BSA and 0.1% Na\(_2\) to remove any remaining small molecular compounds. Four-milliliter fractions were collected, and fractions 10 to 13, representing the main peak of the pure enzyme activity, were chosen as a label in the ELISA.

ELISA for Determination of Topotecan

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, the well in microtiter plates (Nunc F Immunoplates I; Nunc, Reskilde, Denmark) were coated by loading 150 \( \mu \)l of anti-topotecan IgG (10 \( \mu \)g/ml) in 10 mM Tris–HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM Na\(_2\) and allowed to stand overnight at 4 \( ^\circ \)C. After the plates had been washed twice with 0.5 M phosphate buffer (pH 7.0) containing 10 mM ethylenediaminetetraacetate, 10 mM Na\(_2\) and 0.1% BSA (buffer A), they were incubated with 200 \( \mu \)l of buffer A containing 2% BSA for 20 min at 37 \( ^\circ \)C to prevent non-specific adsorption. The anti-topotecan IgG-coated wells were then filled with 50 \( \mu \)l of either topotecan-treated samples, or PBS as a control, followed immediately by 50 \( \mu \)l of the pooled topote-
can-β-Gal conjugate (diluted 1:300 in buffer A for topotecan). The wells were then incubated for 3 h at 4 °C and once again washed briskly with buffer A.

The activity of the enzyme conjugate bound to each well was then measured by the addition of 125 μl of 0.1 mM 4-methylumbelliferyl-β-D-galactopyranoside in buffer A, followed by incubation of the wells at 37 °C for 90 min. The enzyme reaction was stopped by the addition of 75 μl of 0.5 M glycine NaOH buffer (pH 10.3) to each well, and the resulting 4-methylumbelliferone was measured by spectrofluorometry at wavelengths of 355 nm for excitation and 460 nm for emission using a fluorescence microplate reader (Fluoroskan Ascent, Labsystems, Helsinki, Finland).

**HPLC Method**

The HPLC system consisted of a Shimadzu Model LC-10AT liquid chromatograph equipped with a spectrophotometric detector SPD-10A V and a 4×125 mm Lichrospher 100 RP-18 endcapped (5 μm) column (Merck, Darmstadt, Germany). The column was eluted with 75 mM potassium dihydrogen phosphate containing 0.2% (v/v) triethylamine (adjusted to pH 6.0 with phosphoric acid) and methanol (65:35, v/v) at a flow rate of 1 ml/min, and the eluate was monitored at 230 nm. The retention time and peak height were measured with a Shimadzu C-R7A chromatopac.

A 100 μl serum was mixed with 100 μl of cold acetonitrile (0 °C). After vortex mixing for 10 s and centrifugation at 9500 g for 1 min, the clear supernatant was injected into the HPLC system.

**Pharmacokinetic Evaluation**

Two male Wistar rats weighing 250 g each were administered 1 mg/kg topotecan by i.v. bolus injection into the femoral vein. Blood was obtained from the cervical vein before administration of the drug and at intervals thereafter, and the serum separated immediately after blood sampling by centrifugation for 1 min (9500 g, 4 °C) to prevent the lactone ring from opening or closing. The serum was immediately diluted 1:1 (v/v) with cold buffer A (4 °C) and stored at −80 °C for at most 48 h until assayed for topotecan concentration. For the determination of the analyte as lactone form, sample preparation was performed on an ice-water bath. The serum was diluted with cold buffer A (4 °C) to obtain topotecan concentrations appropriate for their measurement by ELISA as described above. The prepared samples were immediately measured by the ELISA. Serum half-lives were calculated using a non-linear least-squares regression program, MULTI.12)

**RESULTS**

**Stability of Topotecan and Its Carboxylate**

The stability of the topotecan and its carboxylate (40 μg/ml) was tested in buffer system at different pH and temperatures (Fig. 3). Topotecan was stable for at least 3 h at 4 °C at pH 6.5 and 7.0 (peak heights were greater than 96.9% of the 0-time peak height). The carboxylate form was stable for at least 3 h at 4°C at pH 7.0 and 7.5 (peak heights were greater than 96.6% of the 0-time peak height). In addition, these compounds in the serum samples were stable at 280 °C for at least 48 h after dilution with buffer A (1:1, v/v) (peak heights were greater than 97.1% of the 0-time peak height).

**ELISA**

The optimal quantities and optimal incubation time for each reaction were established. The dose-response standard curves of topotecan obtained in the serum system are shown in Fig. 4. The limits of topotecan detection by ELISA were between 16 pg and 50 ng/ml of topotecan. For practical purposes, the working range was arbitrarily set between 80 and 2000 pg/ml based on the precision and accuracy findings for the ELISA (Table 1), which showed this developed ELISA to be a reproducible technique. Recoveries of four different levels of topotecan ranging from 80 pg to 10 ng/ml were satisfactory, 96.0 to 105.6% (n=5). The coefficients of variation for intra- and inter-assays between topotecan concentrations of 80 pg to 10 ng/ml at four different levels each were 4.6 to 14.8% and 2.9 to 15.7% (n=5 for each), respectively.

**Specificity**

The antibody specificity was determined by...
the displacement of bound topotecan–β-Gal by other similar compounds. Values of the cross-reactivity were defined as the ratio of each compound to topotecan in the concentrations required for 50% inhibition of topotecan–β-Gal binding to the antibody. The anti-topotecan antibody showed 105.9% cross-reaction with SN-38, 70.4% with camptothecin, 2.2% with the carboxylate form of topotecan and 0.25% with camptothecin-21-isopropylamide (Table 2).

**Comparison of ELISA and HPLC** The ELISA method was compared with an HPLC method by using specific quantities of the lactone form of topotecan in human serum. Ten samples of various concentrations of topotecan ranging from 10 to 80 µg/ml were incubated with human serum for 24 h at 25 °C. The lactone form to carboxylate form concentration ratios ranged from about 0.1 to 0.5. The HPLC technique analyzed the 10 samples, showing a linear relationship between the peak height of the topotecan chromatogram and the injected topotecan dose. ELISA determination was carried out using these topotecan samples, properly diluted to the drug-concentration range detectable by ELISA. Figure 5 shows that there was a good correlation between the values determined by the two methods, and the plot was linear as predicted by the equation \( Y = 1.03X - 0.59 \), where \( Y \) is the concentration value determined by HPLC analysis and \( X \) is that determined by ELISA; the correlation coefficient was 0.989 \((n=10)\).

**Quantification of Topotecan in Rat Serum by ELISA** Two rats, each weighing 250 g, were administered 1 mg/kg topotecan by rapid i.v. injection into the femoral vein. Blood was obtained from the same femoral vein before administration of the drug and at different intervals thereafter, and the topotecan content in the serum was determined by this ELISA. The disappearance of topotecan immunoreactivity from the serum is shown in Fig. 6, demonstrating that a topotecan dose of as little as 23 ng/ml could be quantitatively measured in rat serum by the ELISA. For 5 h after drug administration, the decline of topotecan in serum was well described by a biphasic pattern of pharmacokinetics, with an average serum \( \alpha \)-half-life of 2.3 min and a serum \( \beta \)-half-life of 118.8 min calculated for the two rats, showing that the serum drug level initially declined very rapidly but gradually slowed after administration. Rat urine was also collected and

### Table 1. Precision and Recovery (%) of ELISA for Topotecan

<table>
<thead>
<tr>
<th>Added (pg/ml)</th>
<th>Estimated (pg/ml)</th>
<th>Recovery (%)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80.0</td>
<td>79.6±9.0</td>
<td>99.5</td>
<td>11.3</td>
</tr>
<tr>
<td>400.0</td>
<td>388.0±17.9</td>
<td>97.0</td>
<td>4.6</td>
</tr>
<tr>
<td>2000.0</td>
<td>1970.0±103.7</td>
<td>98.5</td>
<td>5.3</td>
</tr>
<tr>
<td>10000.0</td>
<td>10340.0±1525.8</td>
<td>103.4</td>
<td>14.8</td>
</tr>
<tr>
<td>Inter-assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80.0</td>
<td>81.8±8.1</td>
<td>102.3</td>
<td>9.9</td>
</tr>
<tr>
<td>400.0</td>
<td>393.6±11.6</td>
<td>98.4</td>
<td>2.9</td>
</tr>
<tr>
<td>2000.0</td>
<td>1919.0±88.3</td>
<td>96.0</td>
<td>4.6</td>
</tr>
<tr>
<td>10000.0</td>
<td>10560.0±1656.2</td>
<td>105.6</td>
<td>15.7</td>
</tr>
</tbody>
</table>

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

### Table 2. Specificity of Anti-topotecan IgG

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% cross-reaction (50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topotecan (lactone)</td>
<td>100.0</td>
</tr>
<tr>
<td>Carboxylate</td>
<td>2.2</td>
</tr>
<tr>
<td>SN-38</td>
<td>105.9</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>70.4</td>
</tr>
<tr>
<td>Camptothecin-21-isopropylamide</td>
<td>0.25</td>
</tr>
</tbody>
</table>
assayed for topotecan by the ELISA. The 5 h urine samples from the two animals contained an average of 17.4% of the delivered dose.

DISCUSSION

In general, the antibody specificity on the hapten appears to be towards the group most removed from the region of conjugation to the carrier protein in the immunogen structure. Topotecan has an aromatic hydroxy group in a moiety most removed from its lactone moiety. Therefore, to produce the specific antibody against the lactone form, topotecan immunogen was prepared using diazotized m-ABA with two selective functional groups of diazo and carboxylic acid as a cross-linking agent. The azotized position in the topotecan structure has not been clearly identified, but is predicted possibly to be at the C-11 or C-12 position of the topotecan molecule, judging from the typical azo-coupling reaction of compounds possessing a phenol moiety. The synthesized topotecan–m-ABA was purified by extraction in acidic pH and then conjugated through its carboxyl group to amino groups of protein by the N-succinimidy ester method. The lactone ring of topotecan may be opening, since synthesis and purification of the conjugate are carried out at pH 7.0. Therefore, the pH of purified conjugate was adjusted to 4.0 to convert the carboxylate form into the lactone form. The topotecan–BSA conjugate, with 6.0 mol of topotecan per mol of BSA, induced the formation of a specific antiserum in each of two rabbits immunized.

Topotecan–β-Gal conjugate was also prepared by essentially the same procedure, with DPM structurally different from m-ABA, to eliminate production of antibody binding to the cross-linkage region of β-Gal. The conjugate thus obtained was stable in eluted buffer (pH 6.0) at 4 °C for more than 6 months during which no loss of enzyme or immuno activity was seen.

Under physiological conditions the lactone form of topotecan undergoes a rapid and reversible pH-dependent conversion to a carboxylate form. Therefore, to develop the ELISA for the determination of the lactone form in plasma, it was necessary to find the condition that both the lactone and carboxylate forms were stable. As shown in Fig. 3, these compounds were stable for at least 3 h at 4 °C and pH 7.0. From these findings, the antigen-antibody reaction of this ELISA was performed under pH 7.0 condition at 4 °C for 3 h. The ELISA for topotecan as a lactone form can detect as little as 80 pg/ml of the drug and is reproducible, as indicated by the overall mean coefficient of variation of 8.6% intra-assays and inter-assays at four different levels of topotecan (Fig. 4 and Table 1). The standard curve in the serum system was essentially the same as that in the buffer system.

The specificity of this ELISA is shown in Fig. 5 and Table 2. The cross-reactivities of anti-topotecan antibody with topotecan analogues were examined. The antibody showed high affinity for camptothecin and SN-38. However, the antibody showed very limited reactivity with the other topotecan analogues which opened the lactone ring of topotecan. These findings indicated that the antibody-recognition site was the lactone moiety of the topotecan molecule. Since the antibody recognizes the lactone moiety of topotecan, this ELISA appears to be highly specific against the lactone form. Pharmacokinetic studies of topotecan in humans have previously been reported using HPLC method. Rapid hydrolysis of topotecan lactone results in plasma carboxylate levels exceeding lactone levels as early as 45 min after the start of a 30 min infusion. During the elimination phase in human plasma, the plasma concentration of the carboxylate form is approximately four fold the concentration of the lactone form. Then, using the human serum sample which contains the lactone and carboxylate forms (the lactone to carboxylate concentration ratios ranged from about 0.1 to 0.5), the ELISA method was compared with the HPLC method using specific quantities of the lactone form. There was good correlation; the correlation coefficient was 0.989 between the two methods (Fig. 5). This finding also supports the high specificity of the ELISA.

As a demonstration of the potential of the ELISA, a preliminary pharmacokinetic study of topotecan in rats was performed (Fig. 6). A biphasic pharmacokinetic pattern was observed with a rapid initial clearance followed by a slower elimination phase. The rat serum half-life was calculated to be 2.3 min for the α-phase and 118.8 min for the β-phase. The excretion of topotecan in the urine (based on the total dose administered) was about 17.4% over 5 h as an average
in two rats. The reason for the very rapid initial clearance may be due to a hydrolysis of topotecan lactone in rat serum, judging from the stability of topotecan in plasma.7) The major metabolite of topotecan is its carboxylate form. Other some metabolites of topotecan have already been identified in human plasma and urine.17,18) However, the maximal concentrations of these metabolites detected in human plasma and urine were relatively low.17,18) Therefore, this ELISA may be specific and sensitive enough to quantify topotecan for pharmacokinetic studies in animals and human.

The ELISA presented here is sensitive, specific, reproducible, and adaptable enough for analyses of a large number of samples. The assay should be a valuable tool for use in clinical pharmacological studies.

REFERENCES