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A highly sensitive enzyme-linked immunosorbent assay for etoposide using β-D-galactosidase as a label

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Summary. A highly sensitive enzyme-linked immunosorbent assay (ELISA) for etoposide (EP) was developed, which is capable of accurately measuring as little as 40 pg EP/ml. Anti-EP sera were obtained by immunizing rabbits with EP conjugated with mercaptosuccinyl bovine serum albumin (MS.BSA) using *N*-[β-(4-diazophenyl)ethyl]maleimide (DPEM) as a heterobifunctional coupling agent. An enzyme marker was similarly prepared by coupling EP with β-D-galactosidase (β-Gal; EC 3.2.1.23) via DPEM. This ELISA was specific for EP and showed a very slight cross-reactivity with its major metabolite, *cis*-hydroxy acid of EP (0.91%), but none with 4′-demethylepipodophyllotoxin and drugs commonly used with EP in combination chemotherapy for cancer treatment. The values for EP concentration detected by this assay were comparable with those detected by the high-performance liquid chromatography (HPLC) method. However, the ELISA was about 1,250 times more sensitive in detecting EP at lower concentrations. Using this assay, drug levels were easily determined in the blood and urine of rats for 7 h after i.v. administration of EP at a single dose of 3 mg/kg. Due to its sensitivity and specificity for EP, the ELISA should prove to be a valuable new tool for use in clinical pharmacological studies.

Introduction

Etoposide (EP), a semi-synthetic derivative of epipodophyllotoxin, has been shown to be very effective in the treatment of various forms of human malignant tumors, including testicular carcinoma, small-cell lung cancer, lymphoma, leukemia, CNS tumors, and Kaposi's sarcoma associated with acquired immunodeficiency syndrome (AIDS) [13, 14, 17, 19]. EP has become extensively useful in cancer chemotherapy but causes critical side effects, including leukopenia, alopecia, anorexia, nausea, thrombocytopenia, and fever [16]. Thus, pharmacokinetic studies should be important for its optimal clinical use. Previous pharmacokinetic studies of EP have been undertaken using isotopically labeled EP [2, 5], high-performance liquid chromatography (HPLC) [3, 7], or radioimmunoassay [1, 12]. Non-isotopic enzyme immunoassay of the kind developed for other anti-cancer drugs [9, 10, 20] should be quite useful in these studies.

This paper describes the production in rabbits of an EP-specific antibody, using *N*-[β-(4-diazophenyl)-ethyl]maleimide (DPEM) as a cross-linker for preparation of the immunogen and EP labeled with β-D-galactosidase (β-Gal) as a tracer, and the development of an ELISA capable of detecting as little as 40 pg EP/ml.

Optimal assay conditions were established, and the method was used to determine levels of EP in the serum and urine of rats treated with the drug.

Materials and methods

Materials. EP and 4′-demethylepipodophyllotoxin were obtained from Nippon Kayaku Co., Ltd. (Tokyo, Japan). *cis*-Hydroxy acid of EP was synthesized by the method of Strife and Jardine [21]. N-[β-(4-Aminophenyl)ethyl]maleimide (APEM) was synthesized as previously reported [8]. β-D-Galactosidase (β-Gal; EC 3.2.1.23) from *Escherichia coli* was obtained from Boehringer Mannheim (FRG).

Introduction of the maleimide group into EP. APEM (1 mg, 4.6 μmol) in 25 μl *N*,*N*-dimethylformamide was acidified by the addition of 40 μl 1 *N* acetic acid and then diazotized with sodium nitrite (0.64 mg, 9.2 μmol) in 20 μl distilled water at 0° C for 15 min. Next, 63-μl portions of the above reaction mixture containing DPEM (ca. 3.4 μmol) was added directly to EP (4 mg, 6.8 μmol) in a mixed solution comprising 200 μl *N*,*N*-dimethylformamide and 1.5 ml 0.1 M phosphate buffer (pH 7.0), followed by incubation at room temperature for 30 min with vigorous stirring. The resulting EP-DPEM was extracted with 2 ml ethyl acetate and was used without further purification for preparation of the conjugates using mercaptosuccinyl bovine serum albumin (MS. BSA) as the EP immunogen and β-Gal as the tracer in the ELISA. The yield of EP-DPEM was tentatively estimated to be 31% according to HPLC measurement of the quantity of nonreacted EP. The molar extinction

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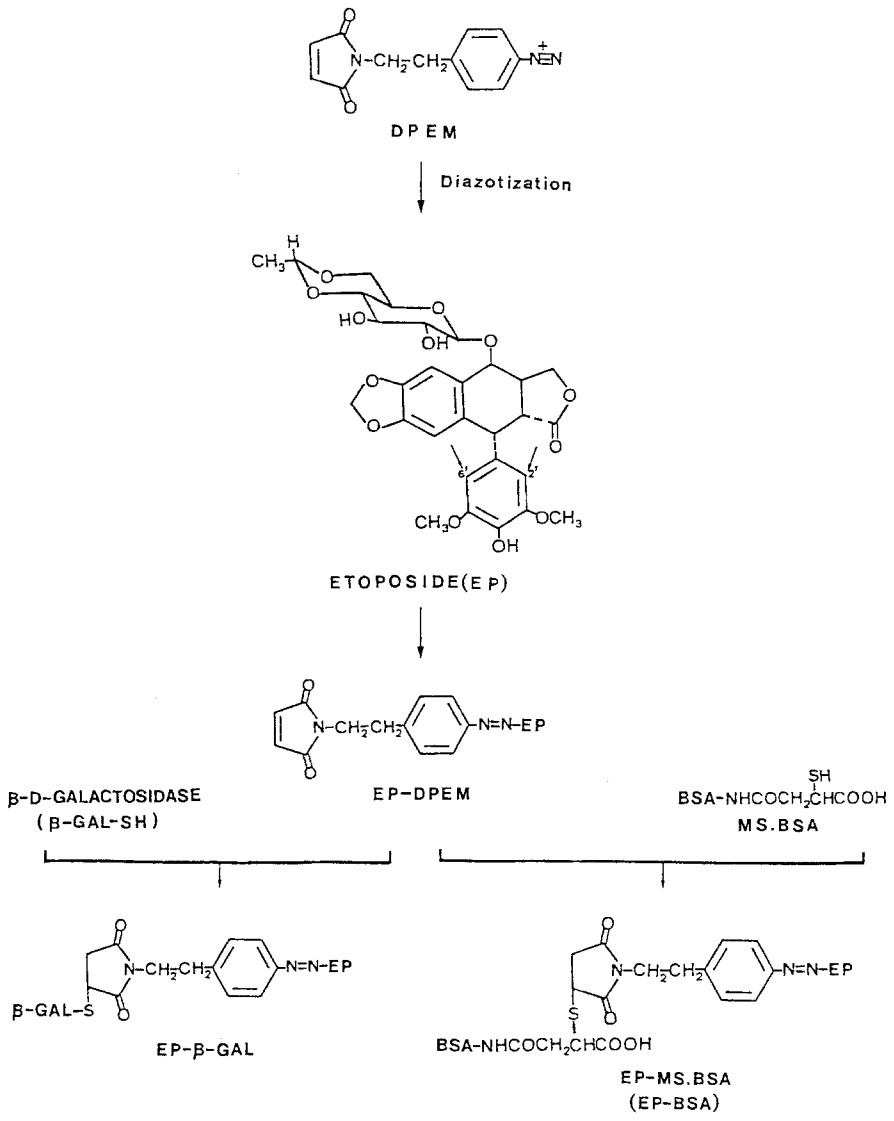

Fig. 1. Scheme for preparation of the immunogen and enzyme conjugate in the ELISA for EP

coefficients of EP-DPEM were thus estimated to be 6,800 at 280 nm and 9,600 at 360 nm, representing those needed to evaluate the quantity of EP conjugated per mole of BSA.

Coupling reaction for hapten. In all, 5 mg acetylmercaptosuccinyl bovine serum albumin (AMS. BSA), containing 17 acetylmercaptosuccinyl groups per BSA molecule, in 0.2 ml 0.1 M phosphate buffer (pH 7.0) was incubated in 50 µl freshly prepared 0.5 M hydroxylamine (pH 7.0) at 25° C for 30 min to remove the protecting acetyl group. After dilution with 1 ml 0.1 M phosphate buffer (pH 7.0) containing 2 M urea, the resulting MS.BSA was immediately added to the EP-DPEM in 200 µl tetrahydrofuran and then incubated at 25° C for 30 min with vigorous stirring. The mixture was chromatographed on a column of Sephadex G-100 (2.8×42 cm) with 0.1 M phosphate buffer (pH 7.0) containing 2 M urea, after which the purified conjugate was examined spectrophotometrically. The latter was estimated to contain about 7.4 molecules of EP per BSA molecule, assuming the molar extinction coefficient of BSA to be 43,600 at 280 nm and those of EP-DPEM to be as described above.

Antibody production in rabbits. An aliquot containing about 1 mg EP-BSA complex was emulsified with an equal volume of Freund's complete adjuvant. Two white female rabbits were each given multiple s. c. injections over sites along both sides of their backs. Booster injections were then given three times at biweekly intervals at one-half the dose used for the first immunization. The rabbits were bled from an ear vein 10 weeks after the start of immunization. The sera (10 ml) were separated by centrifugation and heated at 55° C for 30 min. The crude fraction of IgG was extracted from the sera with 50% saturated ammonium sulfate and then dialysed with 17.5 mM phosphate buffer (pH 6.8) and chromatographed on a column of DEAE-Saphacel (2.1×23 cm) using the same buffer as an eluent [18]. The fraction passed through the column was lyophilized and used as anti-EP IgG for the ELISA.

Preparation of the EP-β-Gal conjugate. EP was labeled by binding to β-Gal, essentially by the same principle used for preparation of the EP immunogen with the aid of DPEM (Fig. 1). In brief, 50 µl tetrahydrofuran solution containing EP-DPEM (approximately 7 µg, 8.5 nmol) was mixed with β-Gal (156 µg, 0.28 nmol) in 1 ml 0.1 M phosphate buffer (pH 6.0), followed by a 30-min incubation at room temperature. The mixture was chromatographed on a column of Sepharose 6B (2×35 cm) using 20 mm phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA, and 0.1% NaN_3 (Buffer A) to remove any small molecular compounds remaining. The eluate was collected in 3-ml fractions and assayed for immunoreactive enzyme activity by the ELISA procedure in the absence or presence of 2 ng free EP/ml. As depicted in Fig. 2, all enzyme activity eluted between fractions 18 and 23, and about 60% of the enzyme activity was inhibited by the presence of 2 ng free EP/ml. Thus, fractions 19–21, representing the main peak of β-Gal activity, were chosen as a label for the ELISA.

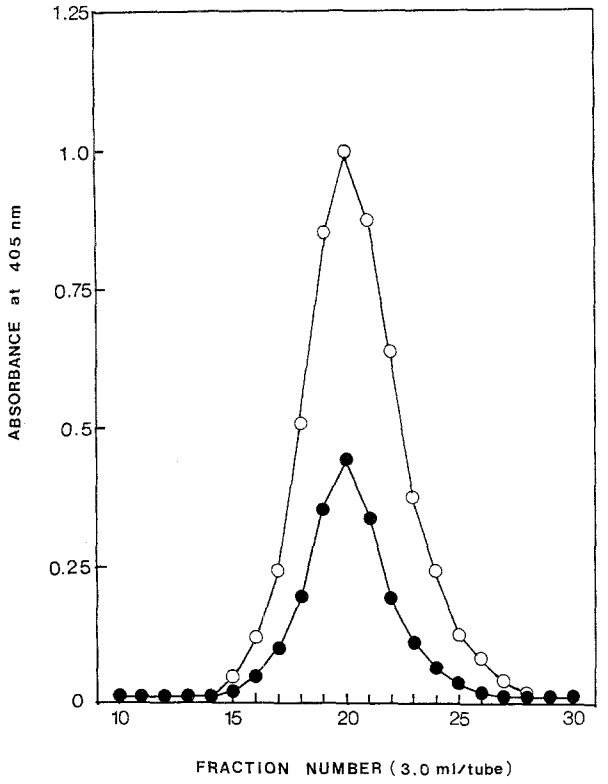

Fig. 2. Elution profile for the EP-β-Gal conjugate from a Sepharose 6B column. Enzyme activity of the conjugate in 2 µl from each fraction was assayed by ELISA in the absence (○) or presence (●) of 2 ng EP/ml

ELISA method. The wells in microtiter plates (Nunc F Immunoplates I; Nunc, Roskilde, Denmark) were coated by loading 150 µl anti-EP IgG (50 µg/ml) in 10 mm TRIS-HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM NaN_3 and allowing this to stand for 20 min at 37° C. After the plates had been washed with 60 mM phosphate buffer (pH 7.4) containing 10 mM ethylenediaminetetraacetate (EDTA) and 0.1% NaN_3 (Buffer B), they were incubated with 200 µl of the same buffer containing 2% BSA for 20 min at 37° C to prevent nonspecific adsorption. The anti-EP IgG-coated wells were then filled with 50 µl of either EP, diluted serum from drug-treated rats, or phosphate-buffered saline as a control, followed immediately by 50 µl of the pooled EP-β-Gal conjugate that had been diluted 1 : 50 in Buffer A. The wells were then incubated overnight at 4° C and once again washed briskly with Buffer B.

The amount of enzyme conjugate bound to each well was then measured by the addition of 125 µl 0.1% 2-nitrophenyl-β-D-galactopyranoside in Buffer A, followed by incubation of the wells at 37° C for a suitable period. Enzymatic activity was stopped by the addition of 75 µl 0.1 M glycine-NaOH buffer (pH 10.3) to each well, and the resulting color intensity was measured spectrophotometrically at 405 nm using an ELISA analyzer (SLT Labinstruments, Austria).

HPLC procedure. The HPLC system consisted of a Shimadzu Model LC-6A liquid chromatograph equipped with a fluorescence detector RF-535 and a 4- × 125-mm Lichrospher 100 RP-18 endcapped (5 µm) column (Merck, Darmstadt, FRG). Various known amounts of EP in 1 ml serum were extracted with 6 ml chloroform by shaking for 10 min. Following brief centrifugation, 5 ml chloroform layer was transferred to a centrifuge tube and evaporated to dryness at 40° C using a nitrogen stream. The dry residue was dissolved in 100 µl methanol. Then, 80 µl solution was injected onto the Lichrospher 100 RP-18 endcapped column, and EP was eluted with a mobile phase of methanol: 0.2 M CH_3COONH_4 (45 : 55, v/v) at a retention time of 5.6 min (flow rate, 1 ml/min) and evaluated in terms of peak height (emission wavelength, 323 nm; excitation wavelength, 292 nm) using a Shimadzu C-R3A Chromatopac.

Pharmacokinetic evaluation. Two male Wistar rats weighing 250 g each were given 3 mg/kg EP 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 was stored at –20° C until assayed for EP concentration. The serum was diluted with Buffer B to obtain EP concentrations appropriate for their measurement by ELISA as described above. Serum half-lives were calculated using a nonlinear least-squares regression program, MULTI [22].

Results

Preparation of immunogen

EP immunogen was prepared by a two-step process using DPEM as a heterobifunctional cross-linking agent (Fig. 1). First, EP was azotized with the agent, and then the resulting EP-DPEM was extracted using ethyl acetate. Thereafter, the maleimide group introduced into an EP molecule was coupled to the thiol groups of MS.BSA by thiolation. After the resulting conjugate (EP-BSA) had been purified by column chromatography on Sephadex G-100, binding of 7.4 mol EP/mol BSA was found.

Labeling of EP

β-Gal labeling of EP was performed at a EP-DPEM: β-Gal molar ratio of about 30 : 1. No significant loss of enzyme activity occurred during the conjugation procedure. The conjugate was stable for up to 3 months in Buffer A at 4° C, with no loss of the enzyme or immunoreactive enzyme activities (data not shown).

Antibody response

Antibodies to EP were produced in each of the two rabbits immunized with the EP-BSA conjugate. The antibody titers in the serum samples were examined by a method similar to that used for anti-actinomycin D antibody [10], and they were found to peak 2 weeks after the third booster injection. The IgG fraction was isolated from the serum and was then used for coating the wells in microtiter plates.

ELISA for EP

This ELISA is based on the principle of competition between enzyme-labeled and unlabeled drug for an immobilized antibody, followed by measurement of the marker enzyme activity of the immunocomplex bound to the solid phase. Optimal quantities and the optimal incubation time for each reaction were established. A standard dose-response curve obtained using Buffer B is shown in Fig. 3. The limits of drug detection by the ELISA were between 20 pg and 20 ng EP/ml; for practical purposes, the working range was arbitrarily set between 40 pg and 12.8 ng/ml based on the precision data for the ELISA (Table 1), which reveal this newly developed ELISA to be a reproducible technique. The intraassay and interassay coefficients of variation between concentrations of 20.4 pg and 12.8 ng

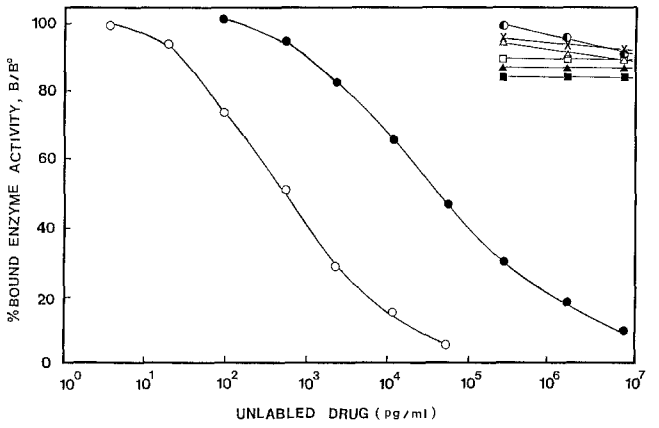

Fig. 3. Standard curve for EP and cross-reactivity of other anticancer drugs with anti-EP antibody. The curves show the amount (percentage) of bound enzyme activity for various doses of EP, 4′-demethylepipodophyllotoxin, *cis*-hydroxy acid of EP, and other drugs (B) as a ratio of that bound using EP-β-Gal alone ($B°$). ○, EP; ◐, 4′-demethylepipodophyllotoxin; ●, *cis*-hydroxy acid of EP; △, Adriamycin; ▲, cyclophosphamide; □, methotrexate; ■, mitomycin C; ×, vincristine

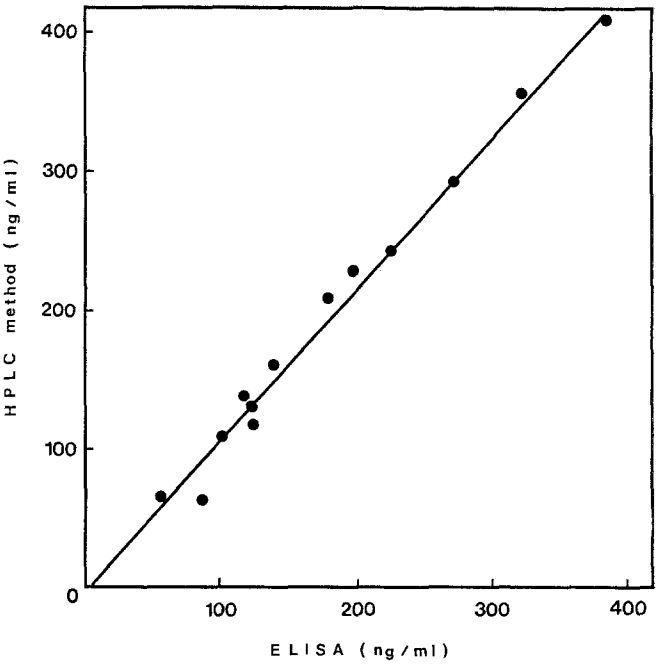

Fig. 4. Correlation of EP quantification by the present ELISA and HPLC methods

Table 1. Precision of ELISA for EP

	Added (pg/ml)	Estimated (pg/ml)	CV (%)
Intraassay	20.4	21.4± 6	28
	102.4	101.6± 10	10.2
	512	518 ± 46	8.9
	2,560	2,552 ±150	5.9
	12,800	12,220 ±820	6.7
Interassay	20.4	21.6± 6	27.8
	102.4	108.8± 11.4	10.5
	512	532 ± 57.6	10.8
	2,560	2,440 ±167	6.8
	12,800	12,440 ±669	5.4

Values represent the mean ± SD of a total of 5 experiments

EP at five different levels each were 5.9%–28% and 5.4%–27.8%, respectively.

The antibody specificity was determined by measuring the displacement of bound EP-β-Gal by 4′-demethylepipodophyllotoxin, by the aglycone moiety of EP, and by the *cis*-hydroxy acid of EP, its major metabolite. The anti-EP antibody showed 0.91% cross-reactivity with EP *cis*-hydroxy acid at the drug concentration required for 50% inhibition, but no cross-reactivity was seen with 4′-demethylepipodophyllotoxin (Fig. 3).

The effects of other anticancer drugs on the ELISA for EP were also examined. The following drugs had no effect on the binding of EP-β-Gal to the antibody at a concentration of 40 μg/ml: Adriamycin, cyclophosphamide, methotrexate, mitomycin C, and vincristine.

Comparison of ELISA and HPLC

The ELISA method was compared with an HPLC method by the use of specific quantities of EP in human serum. The HPLC technique analyzed 13 samples of various concentrations of EP ranging from 50 to 400 ng/ml, showing a linear relationship between the peak height of the EP chromatogram and the injected EP dose. ELISA determination was done using these EP samples, properly diluted to the drug-concentration range detectable by ELISA. Figure 4 shows that there was good correlation between the values determined by the two methods, and the plot was linear as predicted by the equation Y = 1.11X–6.37, where Y is the concentration value determined by HPLC analysis and X is that determined by ELISA; the correlation coefficient was 0.993 (n = 13).

The practical minimal sensitivity of HPLC analysis was also evaluated using specific quantities of EP in human serum and was demonstrated to be 50 ng/ml EP at the high level of the monitor. The signal-to-noise ratio at that sensitivity was approximately 3:1. As the sensitivity of ELISA is known to be at least 40 pg/ml, the HPLC assay was 1,250 times less sensitive.

Quantification of EP in rat serum by ELISA

Two rats, each weighing 250 g, were given 3 mg/kg EP 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 EP content in the serum was determined by this ELISA. The disappearance of EP immunoreactivity from the serum is indicated in Fig. 5, demonstrating that an EP dose of as little as 0.1 μg/ml can be quantitatively measured in rat serum by the ELISA. For 7 h after drug administration, the decline of EP in serum was well described by a biphasic pattern of pharmacokinetics, with an average serum α-half-life of 7.8 min and a serum β-half-life of 73.1 min calculated for the two rats, showing that the serum drug

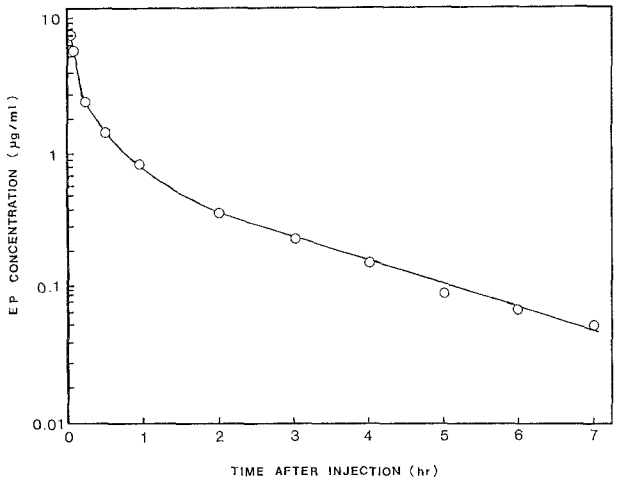

Fig. 5. Serum EP levels in rats after a single i.v. injection of the drug. Two rats, each weighing about 250 g, were given injections of 3 mg/kg EP. At each interval, blood was collected and the serum EP was measured by ELISA

level initially declined very rapidly but gradually slowed after administration. Rat urine was also collected and assayed for EP by the ELISA. The 7-h urine samples from the two animals contained an average of 11.9% ± 8% of the delivered dose.

Discussion

A specific and sensitive ELISA for EP was developed that is suitable for studying the pharmacokinetics of the drug. EP immunogen and EP-β-Gal conjugate (as a tracer) were prepared using DPEM with two selective, functional diazo and maleimide groups as a cross-linking agent. This agent has previously enabled us to develop an enzyme immunoassay for the peptide hormone neurotensin [8] and, more generally, to use the method of enzyme-antibody conjugation [11].

In the present study, the site of azo-coupling of EP by DPEM was not strictly identified but was predicted to be at the C-2′ or C-6′ position of the aglycone moiety, judging from the typical azo-coupling reaction of compounds possessing a methoxy moiety. The resulting EP-DPEM was conjugated by thiolation with the thiol groups of MS.BSA (immunogen) and β-Gal (enzyme marker). The EP-DPEM was found to be very stable in anhydrous acetate and can be stored for >10 months at –20° C, during which time the maleimide group is fully maintained. Therefore, a major advantage of using EP-DPEM is that it can easily be used for conjugation with proteins containing thiol groups whenever such conjugates are needed. This is especially true when preparation of the enzyme conjugate EP-β-Gal with fully immunoreactive enzyme activity is required for an ELISA (Fig. 1). The present conjugation method is simple, mild, and reproducible and does not result in extensive (intra- or intermolecular) self-coupling of EP or proteins, and protein conjugates thus prepared can easily and rapidly be purified by means of gel filtration.

An optimal assay procedure was established for the EP ELISA that proved to be so sensitive that EP concentrations of <40 pg/ml could be measured reproducibly (Fig. 3, Table 1). This sensitivity appears to be 1,250 and 125 times more sensitive than the present HPLC procedure and the previous radioimmunoassay [1] for EP, respectively.

The antibody specificity was directed mainly toward EP, although there is a negligible cross-reactivity (0.91%) with the *cis*-hydroxy acid of EP, a major metabolite seen in plasma and urine [4, 6, 12], and no cross-reactivity with 4′-demethylepipodophyllotoxin, the aglycone moiety of the parent drug. It might therefore be said that the antibody-recognition site is at the sugar moiety and at the lactone ring of the aglycone moiety of the EP molecule. On the other hand, Aherne and Marks [1] and Ho et al. [12] have reported a radioimmunoassay for EP that shows high cross-reactivity of the antibody with the *cis*-hydroxy acid of EP.

Anti-EP antibody showed no cross-reaction with various other anticancer drugs such as Adriamycin, cyclophosphamide, methotrexate, mitomycin C, and vincristine, indicating that the ELISA for EP is appropriate for use in clinical evaluation of combination chemotherapy, since EP is often used with other chemotherapeutic agents (Fig. 3).

The utility of the ELISA was demonstrated by the measurement of serum levels of EP in rats after i.v. administration. A biphasic pharmacokinetic pattern was observed, with rapid initial clearance being followed by a slower elimination phase (Fig. 5). The excretion of EP equivalents in the urine (based on the total dose delivered) was found to average 11.9% over 7 h for two rats. This excretion value might be attributed predominantly to the amount of non-metabolized EP in vivo, since in the present ELISA almost no cross-reactivity was seen with the major EP metabolite, the *cis*-hydroxy acid of EP, or 4′-demethylepipodophyllotoxin, as described above. However, the metabolic fate of EP has not yet been fully elucidated [4, 6, 12].

Our data show that the ELISA for EP, developed using a new coupling agent, DPEM, may be superior to existing radioimmunoassays in both sensitivity and specificity [1, 12]. These advantages of the ELISA may enable clinical pharmacological studies to be carried out during clinical trials of EP, since the drug is commonly used at a relatively high therapeutic dose of 110–130 mg/m^2 daily when given p.o. or 80–100 mg/m^2 daily when given i.v. for 5 consecutive days [15].

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