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Measurement of plasma etoposide by radioimmunoassay

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Abstract: A sensitive radioimmunoassay (RIA) for the specific determination of 4′-demethylepipodophyllotoxin-1β(4,6-O-ethylidene)-α-D-glucopyranoside (etoposide) in human plasma is described. In order to obtain a specific antisera, etoposide–acetonyl-carboxymethoxime–bovine serum albumin (BSA) and etoposide–hemisuccinate–BSA conjugates were synthesized as antigenic hapten carrier proteins. Antisera raised against both conjugates in rabbits and sheep exhibited high specificity and made it possible to discriminate etoposide from picro-etoposide and picro-hydroxy acid. Good correlation was found in a comparison of the new RIA method with one method involving high-performance liquid chromatography ($r = 0.9792$). The method was applied to the direct analysis of etoposide in plasma obtained from cancer patients.

Keywords: *Radioimmunoassay; Etoposide; Etoposide–BSA conjugates; High-performance liquid chromatography.*

Introduction

In order to study the pharmacokinetics of 4′-demethylepipodophyllotoxin-1β(4,6-O-ethylidene)-β-D-glucopyranoside (etoposide) as a therapeutic agent for neoplastic disease [1–4], it is necessary to establish a practical analytical method for the trace amounts of the drug in biological samples.

Several methods for quantitation of etoposide and its metabolites in biological samples have been developed including assay by measurement of total radioactivity [5], and high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [6–8], fluorimetric detection [9] and electrochemical detection [10]. Apart from methods involving radioactivity most are tedious and time-consuming, because the purification process based on liquid–liquid extraction frequently results in the use of relatively large amounts of organic solvents which have to be evaporated to dryness and then reconstituted into a small volume for the subsequent analysis.

Recently, Aherene *et al.* have developed a RIA for quantitation of etoposide in plasma using antiserum obtained by giving to sheep, etoposide–BSA conjugate synthesized by use of sodium periodate reaction [11]. However, they found that the

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antiserum recognized the picro-hydroxy acid analogue more than etoposide itself. This may be attributable to the conversion of etoposide to picro-etoposide or picro-hydroxy acid analogues, whose structures are shown in Table 2, under the alkaline conditions of the coupling process.

In order to enhance specificity for the quantitation of etoposide by RIA, two antigenic etoposide–BSA conjugates have been prepared. This paper deals with the synthesis of new haptens, the preparation of anti-etoposide antibodies and the specificity of antibodies for etoposide. The method has been applied to the quantitation of etoposide in plasma.

Experimental

Materials

Etoposide and its related compounds used in this study were synthesized in the authors' laboratories. [Iα-^{3}H]-etoposide (25 Ci mmol^{-1}) was synthesized from [1α-^{3}H]-4′-demethylepipodophyllotoxin by the method of Kuhn *et al.* [12]. 1,1-Dimethoxy-3-butanone, carboxymethoxyamine hemihydrochloride and succinic anhydride were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Bovine serum albumin and Freund's complete adjuvant were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and Gibco Co. (Detroit, MI, USA), respectively. All other chemicals and solvents were of analytical grade.

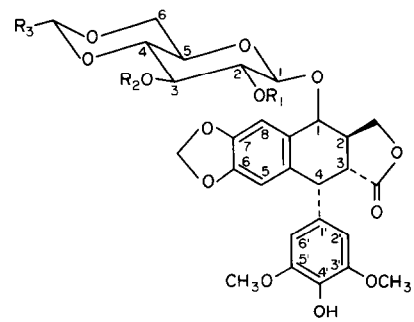
Apparatus

Melting points were measured on a micro hot-stage apparatus and were uncorrected. Field desorption (FD) mass spectrometry was carried out using a JMS DX-300 double focusing mass spectrometer equipped with a FD ion source and a JMA-2000 data processing system (JEOL Ltd., Japan). Emitter current was programmed with a digital emitter current programmer at the rate of 0.1 mA s^{-1}. Nuclear magnetic resonance (NMR) spectra were run on a GX-400 FT-NMR spectrometer (JEOL Ltd., Japan) using tetramethylsilane as internal standard. Abbreviations used: *s* = singlet, *d* = doublet, *t* = triplet, *dd* = double doublet, *q* = quartet, *m* = multiplet.

Synthesis of haptens

Etoposide acetonylcarboxymethoxime (2b). To a solution of 4′-demethylepipodophyllotoxin-1-α-D-glucopyranoside (100 mg) in CH_3NO_2 (5 ml) was added freshly distilled 1,1-dimethoxy-3-butanone (2 ml) and *p*-toluenesulfonic acid (50 mg) and the resulting mixture stirred at room temperature for 2 h. After diluting with $CHCl_3$, the organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. Purification of the resulting crude product by silica gel column chromatography using $CHCl_3$–MeOH and recrystallization from acetone-*n*-hexane (98:2) gave the acetonyl derivative (2a, 65 mg) as colourless prisms: mp 206–209°C. FD-MS: *m/z* 630 ([M]$^{+\cdot}$, I% = 100). NMR ($CDCl_3$) δ: 2.22 (3H, *s*, $-CH_2COC\underline{H}_3$), 2.83 (2H, *d*, $-C\underline{H}_2COCH_3$), 3.76 (6H, *s*, 2 × $-OCH_3$), 5.03 (1H, *s*, $CH_3COCH_2C\underline{H}O_2$), 5.98 (6H, *s*, $-OCH_2O-$), 6.27 (2H, *s*, C2′H and C6′H), 6.55 (1H, *s*, C5-H), 6.82 (1H, *s*, C8-H).

To a solution of (2a, 50 mg) in dry pyridine (2 ml) was added carboxymethoxyamine hemihydrochloride (100 mg) and the resulting solution was stirred at 60°C for 2 h. Purification of the reaction mixture with Sephadex LH-20 column chromatography using $CHCl_3$–*n*-hexane–MeOH (5:5:1, v/v) gave a compound (2b, 35 mg) as colourless prisms



	R_1	R_2	R_3
I (Etoposide)	H	H	CH_3
2a	H	H	CH_2COCH_3
2b	H	H	$CH_2C(CH_3)=N-OCH_2COOH$
3	3a+3b		
3a	$COCH_2CH_2COOH$	H	CH_3
3b	H	$COCH_2CH_2COOH$	CH_3

Scheme 1
Structure of etoposide and its derivatives.

after recrystallization from acetone–*n*–hexane: mp 190–194°C. FD-MS: *m/z* 742 ($[M + K - H]^{+\cdot}$, I% = 47), *m/z* 726 ($[M + Na - H]^{+\cdot}$, I% = 100). NMR ($CDCl_3$) δ: 1.98 (3H, *s*, $-CH_2CNCH_3$), 2.58 (2H, *d*, $-CH_2CNCH_3$), 2.85 (2H, broad *s*, = N–OCH_2COO–), 3.77 (6H, *s*, 2 × $-OCH_3$), 5.97 (6H, *s*, $-OCH_2O$–), 6.25 (2H, *s*, C2′H and C6′H), 6.54 (1H, *s*, C5-H), 6.81 (1H, *s*, C8-H).

Succinylation of etoposide. To a solution of etoposide (300 mg) in dry pyridine (1 ml) was added succinic anhydride (92 mg, 1.8 eq) and the resulting solution was refluxed for 5 h. Purification of the reaction mixture by Sephadex LH-20 column chromatography using $CHCl_3$–*n*-hexane–MeOH (5:5:1, v/v) gave the mono-succinylated derivatives as a colourless oil mixture of positional isomers (3, 200 mg). This mixture (94 mg) was submitted to preparative TLC using $CHCl_3$–MeOH–AcOH (90:10:1, v/v) as developing solvent. Elution of the adsorbent corresponding to the spot with R_f = 0.58 gave a major component (3a, 55 mg) which formed colourless needles after recrystallization from methanol–benzene: mp 203–207°C. FD-MS: *m/z* 688 ($[M]^{+\cdot}$, I% = 39), *m/z* 588 ($[M-(HO_2CCH_2CH_2CO)+H]^+$, I% = 100). NMR ($CDCl_3$) δ: 1.38 (3H, *s*, ethylidene-CH_3), 2.42–2.58 (4H, *m*, $-COCH_2CH_2CO$–), 3.75 (6H, *s*, 2 × $-OCH_3$), 3.81 (1H, *t*, pyranose-C3-H), 4.70 (1H, *d*, pyranose-C1-H), 4.75 (1H, *q*, ethylidene-H), 4.87 (1H, *t*, pyranose-C2-H), 5.85 (2H, *d*, $-OCH_2O$–), 6.25 (2H, *s*, C2′H and C6′H), 6.54 (1H, *s*, C5-H), 6.75 (1H, *s*, C8-H).

Elution of the adsorbent corresponding to the spot with R_f = 0.63 gave a minor component (3b, 24 mg) forming colourless prisms after recrystallization from methanol–benzene: mp 161–164°C. FD-MS: *m/z* 688 ($[M]^{+\cdot}$, I% = 50), *m/z* 588 ($[M-(HO_2CCH_2CH_2CO)+H]^+$, I% = 100). NMR ($CDCl_3$) δ: 1.34 (3H, *d*, ethylidene-CH_3), 2.65–2.75 (4H, *m*, $-COCH_2CH_2CO$–), 3.52 (1H, *dd*, pyranose-C2-H), 3.75 (6H, *s*, 2 × $-OCH_3$), 4.68 (1H, *d*, pyranose-Cl-H), 4.70 (1H, *q*, ethylidene-H), 5.13 (1H, *s*,

pyranose-C3-H), 5.97 (2H, *d*, $-OCH_2O-$), 6.24 (2H, *s*, C2'H and C6'H), 6.55 (1H, *s*, C5-H), 6.82 (1H, *s*, C8-H).

Preparation of conjugate

To a solution of (2b) (100 mg) in dioxane (0.5 ml) were added tri-*n*-butylamine (0.01 ml) and isobutylchlorocarbonate (0.02 ml). The resulting solution was stirred at 10°C for 30 min, after which was added BSA (100 mg) in a mixture of distillated water (2.2 ml) and dioxane (1.5 ml) containing 1N NaOH (0.04 ml). The reaction mixture was stirred at 10°C for 3 h. The resulting solution was dialysed against water at 4°C overnight, after which lyophilization of the solution afforded BSA–conjugate (90 mg) as a fluffy powder. Etoposide hemisuccinate–BSA conjugate was obtained similarly without separation of positional isomers.

Immunization of animals

The antigen (2 mg) was dissolved in sterile isotonic saline (1 ml) and emulsified with complete Freund's adjuvant (1 ml). The emulsion was injected into the rabbits (New Zealand White, female, 2–2.5 kg body wt) subcutaneously at multiple sites along the back. This procedure was repeated at intervals of 2 weeks for the first month and then once a month. The rabbit was bled 5 days after the final booster immunization.

For the sheep (Suffolk, male, *ca* 100 kg body wt), each antigen (5 mg) was emulsified in saline (2.5 ml) and complete Freund's adjuvant (2.5 ml) with injection intradermally at multiple sites along the back at intervals of one month. The bleeding was done as for the rabbit.

The antisera were separated by centrifugation at 1910 ***g***, 4°C for 10 min, and stored at −50°C. Antisera were used in the appropriate dilution.

Assay procedure

All dilutions of standard, tracer and antisera were made using 0.05 M phosphate buffer (pH 7.3) containing 0.1% (w/v) NaN_3, 0.1% (w/v) rabbit IgG and 0.9% (w/v) NaCl. To a series of standard solutions of etoposide and [1α-^{3}H]etoposide (0.2 ml) was added diluted antiserum (0.1 ml). The mixture was incubated at 30°C for 2 h, and stored at 4°C overnight. After cooling, dextran-coated charcoal suspension in 0.05 M phosphate buffer (0.5 ml) was added; the mixture was vortexed well, allowed to stand at 0°C for 15 min, and centrifuged at 1910 ***g***, 4°C for 15 min. The supernatant was transferred into a plastic counting vial containing Atomlight® (New England Nuclear, USA) scintillant and counted for radioactivity.

Results and Discussion

For the production of specific antisera to etoposide it is necessary to prepare antigenic etoposide–BSA conjugates. Unfortunately, etoposide has no functional group which can be available for coupling to BSA directly and effort was directed to the synthesis of a suitable hapten.

Treatment of the desacetal derivative of etoposide with 1,1-dimethoxy-3-butanone in CH_3NO_2 and *p*-toluene sulfonic acid gave the acetonyl derivative (2a). Condensation of (2a) with carboxymethoxyamine hemihydrochloride in pyridine solution afforded the corresponding carboxymethoxime derivative (2b) in a quantitative yield. Figure 1 shows the field desorption (FD) mass spectrum of (2b). Appearance of the ion at *m/z* 726

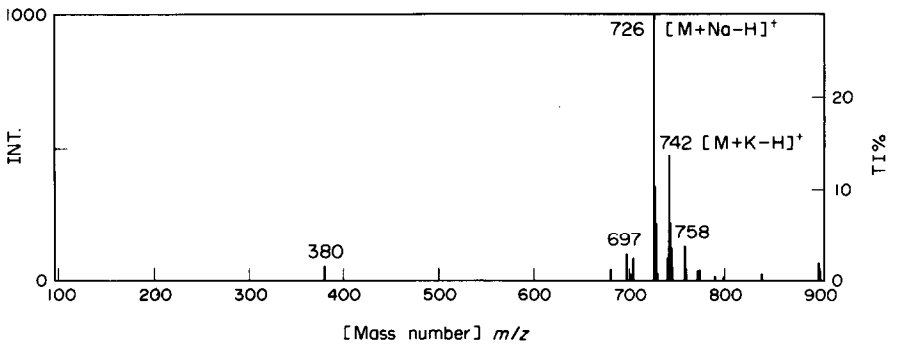


Figure 1
FD mass spectrum of compound (2b) obtained with an emitter current of 21mA.

(M+Na−H) indicated the incorporation of the acetonyl and carboxymethoxyl moiety into the etoposide molecule. Further structural elucidation was done by means of high resolution nuclear magnetic resonance (NMR) spectroscopy.

Esterification of etoposide with slight excess of succinic anhydride resulted in the selective formation of the mono-hemisuccinate. The crude product contained two compounds, assumed to be the positional isomers of the succinyl group. In order to confirm the site of succinyl substitution, the isomers were separated by thin layer chromatography using $CHCl_3$–MeOH–AcOH (90:10:1, v/v) as developing solvent and the chemical shift assignment of protons in the sugar moiety was carried out by high resolution NMR spectroscopy using the homonuclear decoupling technique. The major isomer of hemisuccinate produced a substantial downfield chemical shift in the proton resonance of pyranose C-3 positions relative to its position on etoposide. On the other hand the minor isomer produced similar downfield chemical shift in the proton resonance of pyranose C-2 position [13]. From these results, the actual position of succinylation in the major and the minor isomers was confirmed at pyranose C-3 and C-2 positions, respectively. Figure 2 shows the FD mass spectrum of the major isomer of hemisuccinate. The molecular ion at *m/z* 688 readily lost the succinyl moiety to give the ion at *m/z* 588 as a base peak. The appearance of the molecular ion and the ion of *m/z* 588 was sufficient to confirm the structure of hemisuccinate.

For the synthesis of hapten–BSA conjugate, mono-hemisuccinate derivatives of etoposide were used directly without separation.

Coupling of each hapten to BSA was carried out by a mixed anhydride method and yielded the corresponding etoposide–BSA conjugates. The number of haptens bound into BSA was determined using UV spectroscopy. The spectral measurement revealed that 22 moles of (2b) and 19 moles of (3) were linked to each BSA molecule.

Rabbits and sheep were immunized with these hapten–BSA conjugates emulsified in complete Freund's adjuvant. Serum samples obtained from these animals exhibited remarkably increased immunoreactivity to etoposide (Table 1). Judged from the Scatchard plot, antisera raised against rabbits and sheep exhibited extremely high affinity for etoposide with an association constant of $1.46–11.9 \times 10^9$ per mol [14].

The calibration curve for etoposide was constructed with the final dilution of 1 to 12,000 using antiserum Re. It will be seen from Fig. 3 that the assay can detect etoposide over a range of 30 to 1000 picograms. When logit transformation was used to reconstruct

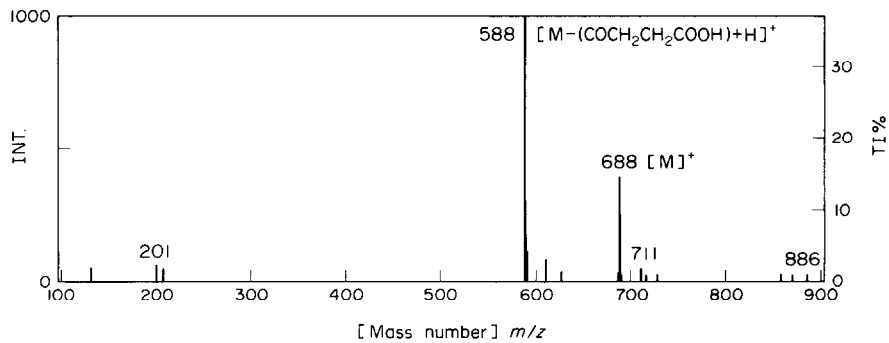


Figure 2
FD mass spectrum of compound (3a) obtained with an emitter current of 18mA.

Table 1
Characterization of antisera obtained from rabbits (Ra–Rg) and sheep (Sa and Sb)

Antisera	Titre	Association constant Ka (M^{-1})
Ra	1: 8000	8.15×10^9
Rb	1:14 000	6.97×10^9
Rc	1: 7300	11.9×10^9
Rd	1:23 000	3.79×10^9
Re	1: 9200	2.68×10^9
Rf	1:23 500	1.55×10^9
Rg	1:25 000	1.46×10^9
Sa	1: 400	*
Sb	1:12 800	2.69×10^9

*Not tested.

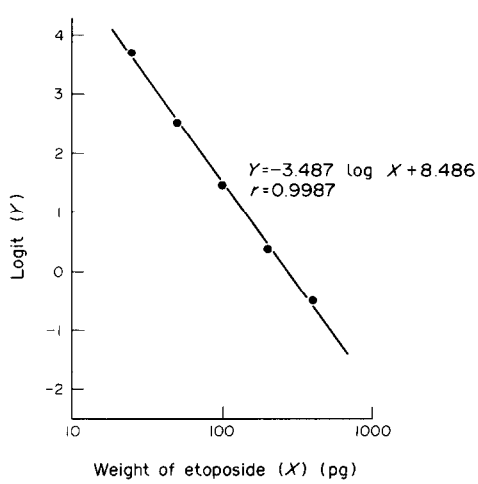
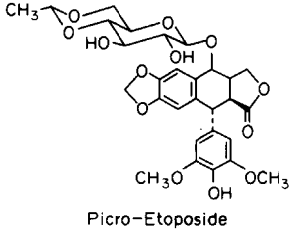


Figure 3
Typical standard curve obtained with a dilution of 1:12 000 using antiserum Re, ^{3}H-etoposide (*ca* 20 000 dpm, 250 pg) and unlabelled etoposide (30–500 pg). Values are the mean of duplicate determinations expressed after log–logit transformation.

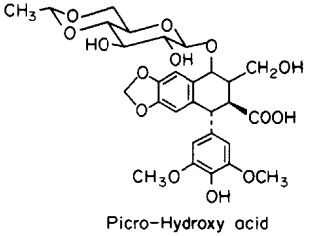
Table 2
Specificities of individual antisera obtained from rabbits (Ra–Rg) and sheep (Sa and Sb)

	Cross reactivity (%)								
	Antisera Ra	Rb	Rc	Rd	Re	Rf	Rg	Sa	Sb
Etoposide	100	100	100	100	100	100	100	100	100
Picro-etoposide	<0.05	<0.05	0.15	0.10	<0.05	<0.05	<0.05	0.42	<0.05
Picro-hydroxy acid	0.42	0.18	0.05	0.05	<0.05	<0.05	<0.05	0.28	<0.05
Etoposide desacetal	0.40	0.29	4.00	<0.05	37.0	16.0	11.0	11.0	39.0

Antiserum Ra–Rd, Sa: Etoposide hemisuccinate–BSA conjugate. Re–Rg, Sb: Etoposide carboxymethoxime–BSA conjugate.



Picro–Etoposide



Picro–Hydroxy acid

the standard curve, the plot of logit value versus logarithm of the amount of unlabelled etoposide showed good linearity (r = 0.9987).

The specificity of the individual antisera was assessed by testing, with ^{3}H-etoposide, the competition in binding to the antibody between etoposide and its related compounds. The results are listed in Table 2, in which the cross reactivity is expressed as a weight ratio of the test compound to etoposide in the concentration giving a 50% displacement of ^{3}H-etoposide. The results incidate that for all antisera produced in rabbits there is little or no binding with picro-etoposide. The desacetal derivative of etoposide shows relatively higher cross reactivity with antisera Re to Rg than with antisera Ra to Rd. This may be ascribed to a structural feature of the hapten–BSA conjugate coupled through the acetal group through the glucosidic moiety. A similar tendency was recognized in antisera obtained from sheep. Thus, the antisera raised against these hapten–BSA conjugates in rabbits and sheep are more specific for etoposide than antisera obtained by coupling etoposide to BSA under the sodium periodate reaction.

Accuracy of the assay was examined by measuring the recovery of etoposide added to dog plasma. The results shown in Table 3 indicate satisfactory recovery. There was no statistically significant difference in sample preparation.

In order to confirm the validity of the new RIA method, each of the plasma samples was analysed by the technique using antiserum Rc and also by a convenient HPLC method with fluorescent detection developed in our laboratories (column; μ-Bondapak™ phenyl, 30 cm × 3.9 mm i.d., Waters Assoc., mobile phase; methanol–H_2O = 55:45 (v/v), flow rate; 1 ml min^{-1}, fluorimetry; Ex 288 nm, Em 328 nm.). The results were plotted as shown in Fig. 4. A good correlation between the two methods is indicated (r = 0.9792).

Figure 5 shows the plasma levels of etoposide in patients with cancer after intravenous (200 mg man^{-1}) and oral (400 mg man^{-1}) administration of etoposide. In the case of intravenous administration, the plasma level exhibited a bi-exponential decrease with half lives of 1.1 h (α-phase) and 4.2 h (β-phase). After oral administration the plasma concentration reached a maximum of 10.11 μg ml^{-1} at 2 h and decreased gradually with a half life of 3.3 h.

Table 3

(a) Recovery of etoposide added to dog plasma

Added (ng ml^{-1})	Recovered (ng ml^{-1})					Recovery (%)
10.0	10.90	11.50	9.80	10.90	10.50	107.2 ± 6.3
5.0	4.80	5.30	5.35	5.38	5.20	104.1 ± 4.7
2.5	2.35	2.55	2.25	3.15	2.68	103.8 ± 14.1
1.25	1.45	1.40	1.27	1.45	1.48	112.8 ± 6.7

(b) Statistical analysis of variance

	ss	f	mf	Fo
Sample preparation	258.8	3	86.27	1.14
Error	1207.2	16	75.45	
Total	1466.0	19		$F^3_{16}(0.05)$ = 3.24

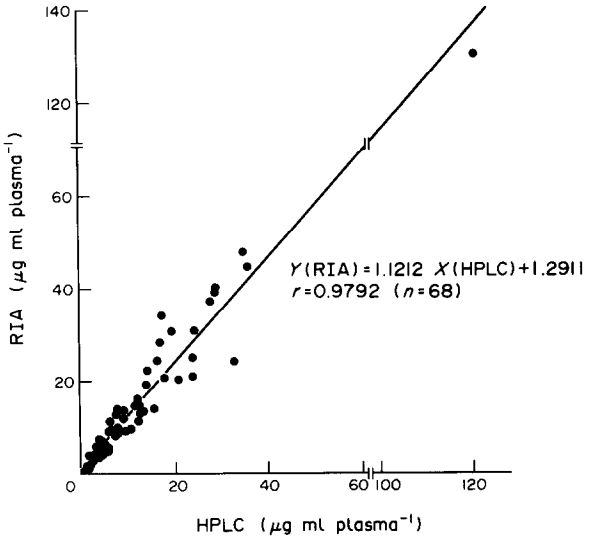


Figure 4
Correlation of plasma etoposide concentration measured by HPLC(X) and RIA(Y).

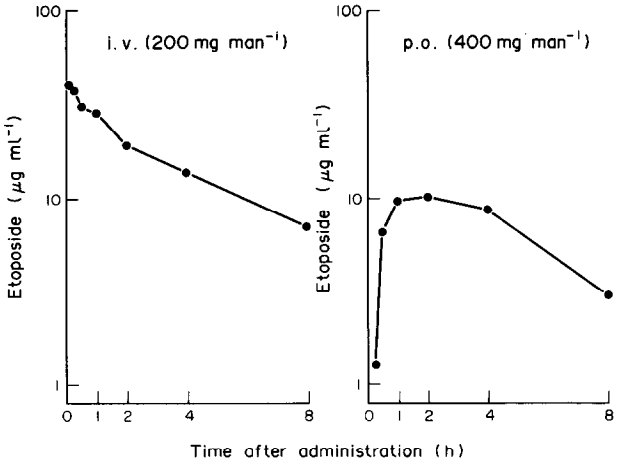


Figure 5
Plasma concentration of etoposide in patients with cancer after intravenous and oral administration of etoposide.

In conclusion, the development of a RIA of etoposide made it possible to enhance the specificity and sensitivity of determination. The availability of the specific antisera described in this paper could eliminate the necessity of extraction and other tedious purification processes even in the presence of picro-etoposide and picro-hydroxy acid.

References

[1] P. J. Creaven and L. H. Allen, *Clin. Pharmacol. Ther.* **18**, 221–226 (1975).
[2] L. M. Allen and D. J. Dreaven, *Eur. J. Cancer* **11**, 697–707 (1975).

[3] M. Rozencweig, D. D. van Hoff, J. E. Henny and F. M. Muggia, *Cancer* **40**, 334–342 (1977).
[4] B. F. Issell, C. Tihon and M. E. Curry, *Cancer Chemother. Pharmacol.* **7**, 113–115 (1982).
[5] F. R. Pelson, L. M. Allen and P. J. Creaven, *J. Pharm. Sci.* **67**, 1106–1109 (1978).
[6] P. Farina, G. Marzillo and M. D'Incalci, *J. Chromatogr.* **222**, 141–145 (1981).
[7] R. J. Strife, I. Jardine and M. Colvin, *J. Chromatogr.* **182**, 211–220 (1980).
[8] J. J. M. Holthuis, H. M. Pinedo and W. J. van Oort, *Anal. Chim. Acta* **130**, 23–30 (1981).
[9] R. J. Strife, I. Jarden and M. Colvin, *J. Chromatogr.* **224**, 168–174 (1981).
[10] J. J. M. Holthuis, F. M. G. M. Römkens, H. M. Pinedo and W. J. van Oort, *J. Pharm. Biomed. Anal.* **1**, 89–97 (1983).
[11] G. W. Aherne and V. Marks, *Cancer Chemother. Pharmacol.* **7**, 117–121 (1982).
[12] M. Kuhn and A. von Wartburg, *Helv. Chim. Acta* **51**, 1631–1641 (1968).
[13] C. F. Brewer, J. D. Loike, S. B. Horwitz, H. Sternlicht and W. J. Gensler, *J. Med. Chem.* **22**, 215–221 (1979).
[14] G. Scatchard, *Ann. N. Y. Acad. Sci.* **51**, 660–672 (1949).

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