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Current Status of Bioanalysis of Etoposide and Related Compounds(1)

KUO-HSIUNG LEE AND HUI-KANG WANG

Natural Products Laboratory, Division of Medicinal Chemistry and Natural Products,
School of Pharmacy, University of North Carolina,
Chapel Hill, North Carolina 27599

ABSTRACT

Etoposide (VP16 or VP16-213) which was approved by the United States FDA for testicular cancer in 1983, is one of the most effective anticancer agents in the treatment of testicular teratoma, Hodgkin's and non-Hodgkin's lymphomas, small-cell lung cancer, and a variety of other malignancies. Development of bioanalytical methods for VP16 and its metabolites is critical for pharmacokinetic and metabolic studies as well as for mechanisms of action studies. In this review, the current status of the bioanalysis of VP16 and its metabolites are discussed. Recent progress in mechanism of action and metabolism studies is also presented. A number of HPLC methods employing UV, fluorescence, or electrochemical detection are available for the bioanalysis of VP16 and its metabolites. Most of these HPLC methods are sensitive and reliable enough for determining the parent drugs and their metabolites in biological fluids. In addition to radioimmunoassay, enzyme-linked immunosorbent assay (ELISA) for VP16, which is the most sensitive assay method to date, has been developed based on modern immunoassay technology. The primary pharmacological and pharmacokinetic data of GL331, a new derivative of epipodophyllotoxin discovered and developed in author's laboratory, which has shown superior antitumor activity to that of VP16, are also discussed.

Key words: Etoposide, VP16, teniposide, VM26, podophyllotoxin, etoposide phosphate, NK611, GL331, antitumor, bioanalysis, HPLC, RIA, ELISA.

INTRODUCTION

Etoposide (VP16 or VP16-213) and teniposide (VM26), are both semisynthetic analogs of podophyllotoxin, an antitumor natural product from certain plants of *Podophyllum*, berberidaceae. The aqueous extracts of the roots or rhizomes of *Podophyllum* were called podophyllin and included in the first U.S. Pharmacopoeia (U.S.P. 1820). It was used hundreds of years ago by the American Indians and by natives of the Himalayan mountains as a cathartic and anthelmintics. The American colonists subsequently used podophyllin as an emetic. The antimitotic properties of podophyllin were first discovered in 1946, and chemical analysis of podophyllin revealed a number of cytotoxic lig-

Correspondence to:Kuo-Hsiung Lee

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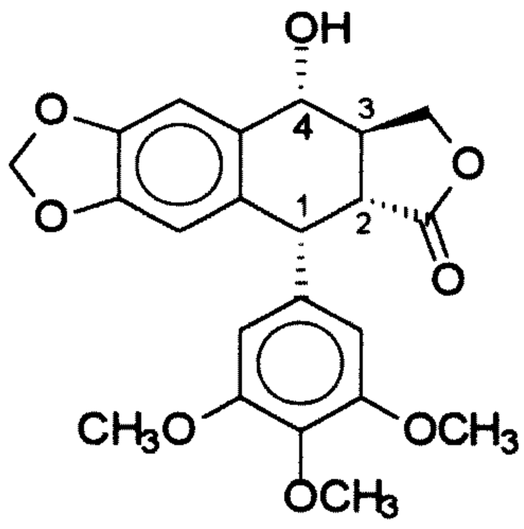
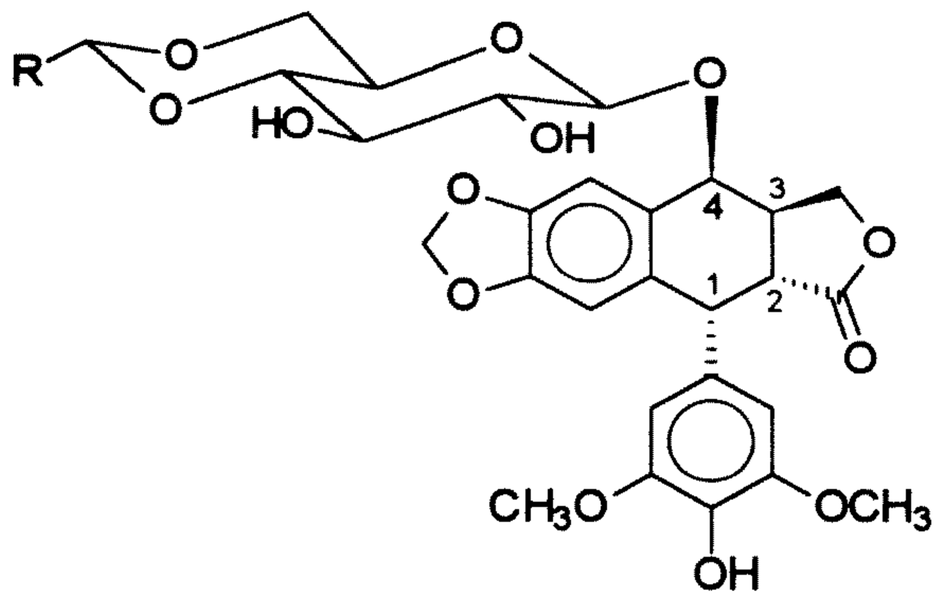
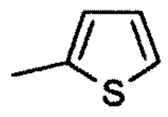


Podophyllotoxin



Etoposide (VP16) : R = CH_3

Teniposide (VM26) : R = 

nan compounds including podophyllotoxin. Due to the high toxicities of these natural products, Sandoz initiated a semisynthetic podophyllotoxin derivative program. The two most successful anticancer analogs discovered from this program were VP16 and VM26. VM26 was synthesized in late 1965; VP16 about nine months later. VM26 was first tested in man in 1967, and VP16 in 1971. The two compounds were licensed to the US company Bristol-Myers in 1978, even though Sandoz had already commercialized VM26 in some countries in 1976 under the names of Vumon® and Vehem®.

Etoposide, which was approved by the United States Food and Drug Administration for treating testicular cancer in 1983 and introduced in the US market as VePesid® by Bristol-Myers, is one of the most effective anticancer agents in the treatment of testicular teratoma, Hodgkin's and non-Hodgkin's lymphomas, small-cell lung cancer, and a variety of other malignancies. Both VP16 and VM26 appear to be useful compounds in the treatment of certain tumors. Although they differ somewhat in their pharmacokinetic profiles, there is no evidence that one drug is superior to the other in a specific tumor type. Their clinical toxicities are similar as well[2].

A number of reviews have been published which cover all aspects of VP16 and VM26 including their chemistry, biology, pharmacokenetics, and clinical applications[3-13]. Among these papers, two excellent reviews written by Stähelin and Wartburg detailed the discovery of etoposide and the development of podophyllotoxin derivatives[10-11]. Our recent review summarized hundreds of newly synthesized VP16 analogs and their biological evaluation[13]. Therefore, this review will assess only the most recent progress in the research on mechanism of action, metabolism and development of bioanalytical methods for VP16 and VM26. Finally, the primary pharmacological and pharmacokinetic data of GL331, a new derivative of epipodophyllotoxin discovered and developed in author's laboratory, which has shown superior pharmacological profiles as an antitumor agent to that of VP16, will also be discussed.

MECHANISM OF ACTION

The previous investigations revealed that the epipodophyllotoxins interact with DNA topoisomerase II and might affect the breaking-re-joining reaction of mammalian DNA topoisomerase II by trapping a covalent enzyme-DNA cleavable complex. The most recent studies have shown

that the mechanism of action of etoposide could involve its bioactivation to metabolites that can damage DNA. Active metabolites of etoposide, generated *in vitro*, are the 3',4'-dihydroxy-derivative (catechol) and its oxidation product, the *ortho*-quinone. The conversion of the catechol to the *ortho*-quinone (and vice versa) proceeds *via* formation of a semi-quinone free radical. Sinha *et al* indicated that both VP16 and VM26 form oxygen-centered phenoxy free radicals during *in vitro* horseradish peroxidase (HRP)/H_2O_2 and prostaglandin H synthase (PGS)/arachidonic acid (AA) activation and that this peroxidative activation results in the formation of two metabolites. Using HPLC and mass spectrometric analysis, one metabolite has been identified as the *ortho*-quinone derivative of the parent drug, which results from peroxidase-mediated O-demethylation[14]. Mans *et al* [15] investigated the role of this radical species in the inactivation of biologically active single- (ss) and double-stranded (RF) Φ X 174 DNA. Their data showed that the primary semi-quinone free radical of etoposide may, to a great extent, be responsible for the *ortho*-quinone-induced ss Φ X 174 DNA inactivation, but that this radical species is not lethal towards RF Φ X 174 DNA.

Later, Sakurai and Lee *et al* demonstrated that podophyllotoxin (PD), etoposide, 4'-demethylepipodophyllotoxin (DEPD), and syringic acid (SA) exhibit no DNA-cleaving activity but, in the presence of metal ions such as Cu^{2+} and Fe^{3+}, DEPD and SA form metal complexes, which in turn show high activity for DNA

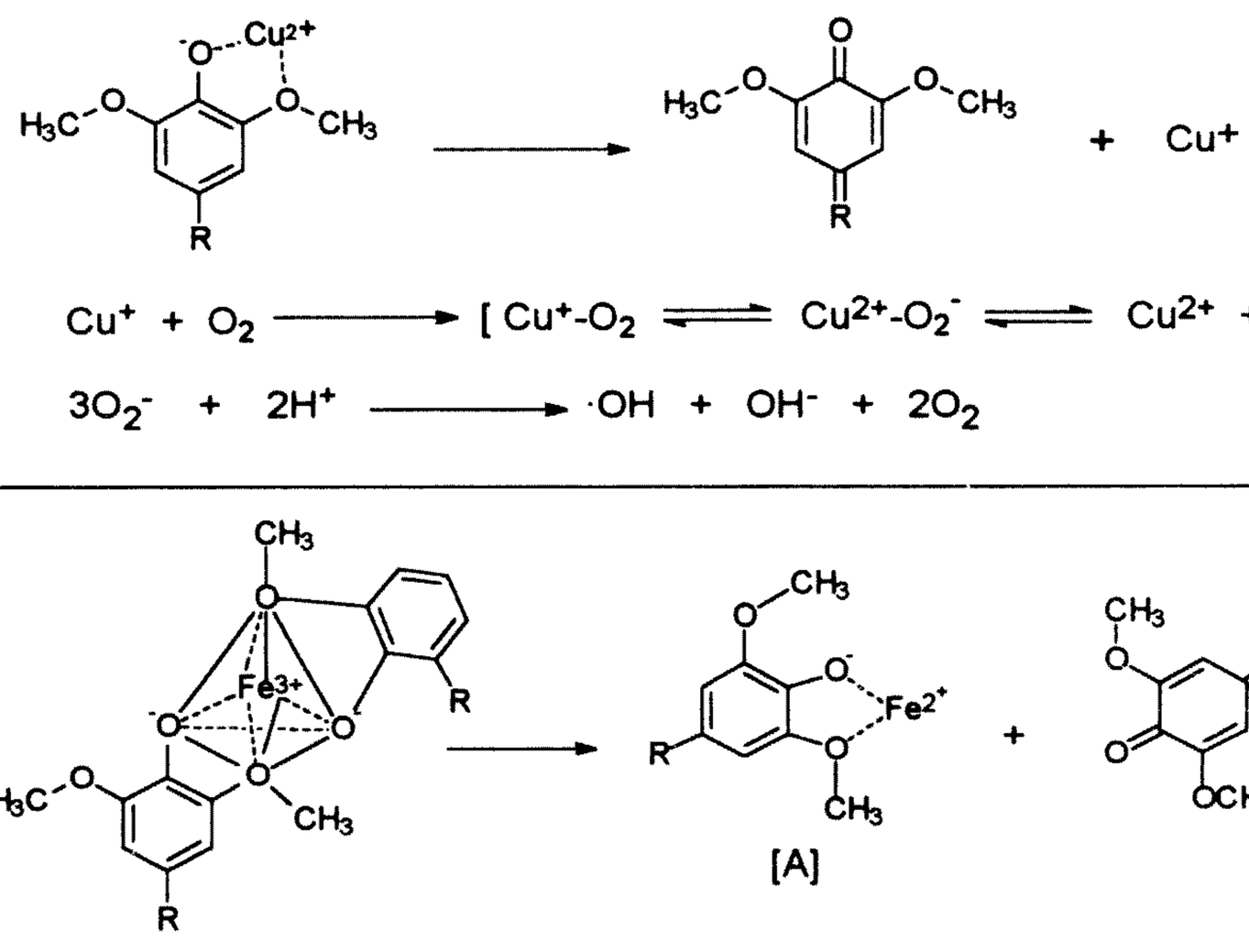


Figure 1. Proposed mechanism for DNA-cleaving activities of the metal complexes

strand scission at pH 7.8 in air[16]. Furthermore, it was found that DNA cleavage was greatly promoted by irradiation with UV light. The PD-Fe^{3+} system at pH 7.8 showed very low DNA-cleaving activity, but irradiation of the system with UV light induced almost complete DNA breakage. In the Cu^{2+}-SA and Fe^{3+}-PD system, with or without UV irradiation, DNA cleavage was significantly inhibited in the presence of hydroxyl radical scavengers, such as sodium benzoate and dimethylurea. These reactions were investigated by optical and electron spin resonance (ESR) spectra, coupled with ESR spin-trapping technique. The formation of hydroxyl radicals was clearly detected in all systems. These findings have led to a new proposal of a metal- and photo-induced mechanism (Figure 1) for the antitumor action of PD, VP-16, and their related compounds. It appears likely that the active hydroxyl radical generated by metal ions is responsible for DNA strand cleavage.

METABOLITES OF VP16 AND VM26

The most important metabolic pathways of VP16 and VP26 include hydrolysis, epimerization, conjugation, demethylation and bioactivated oxidation as shown in Figure 2.

Hydroxy Acid

The main metabolic route for VP16 is believed to be hydrolysis of the lactone ring to the hydroxy acid derivative. Allen *et al* isolated the major urine metabolite of VP16 and identified it as 4'-demethylepipodophyllic acid by mass spectrometric analysis of its methyl ester[17]. This metabolite has also been isolated by other investigators. Strife *et al* isolated the hydroxy acid of VP16 by extraction with XAD-4 resin from plasma of patients treated with VP16[18]. Evans *et al* detected a hydroxy acid derivatives in the plasma and urine of patients with similar retention time

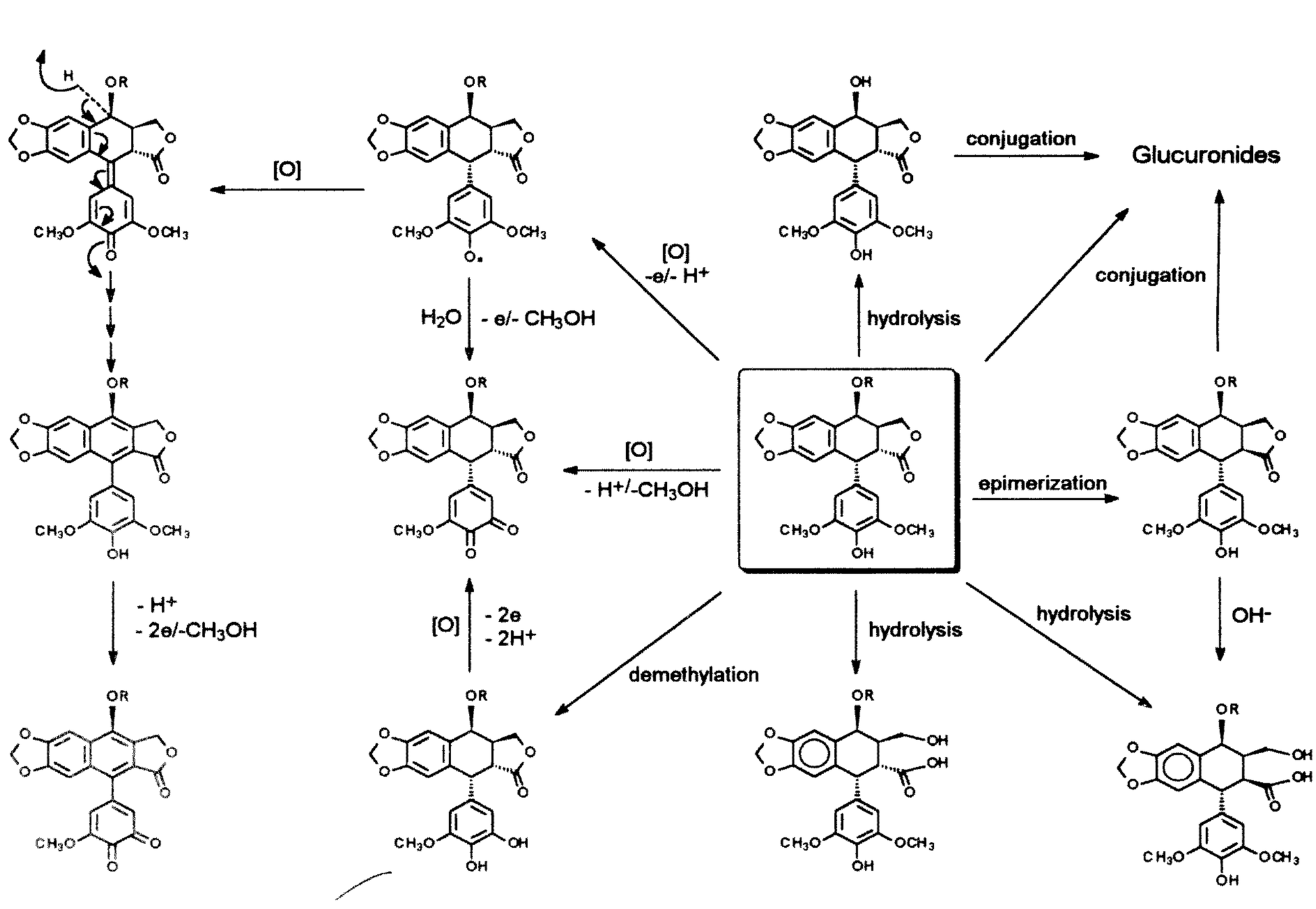


Figure 2. Possible metabolic pathways of VP16 and VM26.

to the synthetic hydroxy acid[19]. Holthuis *et al* isolated a hydroxy acid of VP16 from urine and bile pre-extracted with 1,2-dichloroethane[20]. Ho *et al* used a radioimmunoassay to detect the presence of the hydroxy acid in urine, bile, and tissue following administration of VP16[21]. In a patient who received VP16 (60 mg/m^2 per day, continuously infused in 2.5 h), 15 % and 18.8 % were excreted as VP16 and its hydroxy acid, respectively, in 5.3 h. The metabolic formation of the hydroxy acid derivative should be catalyzed by an esterase, and the metabolite probably possesses the 1,2-*trans* configuration, but unambiguous evidence has not been established. The synthetic *cis*-hydroxy acid was reported to have no cytotoxic effect against human leukemic lymphoblasts[22]. The *trans* hydroxy acid may retain cytotoxic activity, but regretfully, this compound has not yet been synthesized due to epimerization of the lactone ring to the *cis* configuration at pH values above 9.5[18].

Picrolactone (Z-isomers)

The Z-isomers have been detected by HPLC in small amounts in plasma[19,23], serum[19], urine[19], cerebrospinal fluid[24], and liver[25]. VP16 is converted into its Z isomer at elevated pH or temperature, indicating that the conversion can occur by a nonenzymatic mechanism. The Z isomer of VP16 was also found in small amounts when the compound was incubated with rat liver microsomes[26]. The *picro* isomer of VP16 also showed no cytotoxic effect against human leukemic lymphoblasts[22].

Aglycone

This metabolite (4'-demethylepipodophyllotoxin) might be formed by the action of β-glycosidase[23] and may contribute to the cytotoxicities of VP16 and VM26, since it inhibits the microtubule assembly and is active *in vitro*. Holthuis *et al* reported the production of small amounts of the aglycone by rat liver microsomes[27]. The aglycone was not found in the urine of patients treated with VP16[19]. However, Gouyette *et al* observed the presence of the aglycone in plasma[28].

Glucuronide

Conjugation of VP16 with glucuronic acid has been detected in bile by Colombo *et al* [25], and in urine by D'Incalci *et al*[29], and Holthuis[30], and Hande *et al* [31]. Colombo *et al* detected two HPLC peaks corresponding to glucuronides in the bile of etoposide-perfused rat livers. Both peaks disappeared and the VP16 peak concomitantly increased after incubation of bile fractions with β-glucuronidase/arylsulphatase[25]. These studies suggested that the two hydroxy groups in the glucoside moiety were involved in glucuronide formation rather than the glucuronic acid being linked to the phenolic group. In contrast to the findings of Colombo *et al* [25], Holthuis reported that the glucuronide of VP16 isolated from patients' urine was linked at the phenolic C-4' position[30]. Holthuis also observed this glucuronide to be the major metabolite of VP16 instead of the hydroxy acid derivative, which was considered by other investigators to be the major metabolite. Hande *et al* confirmed the formation of the glucuronide of VP16 in the rat and the rabbit by mass spectrometric evidence[31]. D'Incalci *et al* isolated the glucuronide of 4'-demethylepipodophyllotoxin in the urine of a patient who received VM26[32].

Oxidative Products

As mentioned above, the activity of epipodophyllotoxins is due in part to the activity of its oxidative products. The peroxidative activation of VP16 and VM26 has been investigated by Sinha *et al*[14]. Both of these drugs, in the presence of horseradish peroxidase or prostaglandin synthetase, formed phenoxyl radical intermediates, which were confirmed by the ESR spectra. Furthermore, this activation also resulted in the formation of two metabolites from each drug. One metabolite was identified as the reactive

ortho-quinone of the parent drug and resulted from the peroxidative O-demethylation. Later, the 3',4'-dihydroxy derivative (DHVP-16) of VP16 was detected from incubation of liver microsomes and identified by mass spectrometric analysis. The formation of DHVP-16 was cytochrome P-450 mediated as indicated by its dependence on NADPH and required oxygen. ESR studies indicated that the same semiquinone radical was also formed during enzymatic (oxidation or reduction) metabolism of DHVP-16 and the *ortho*-quinone derivative of VP16 by incubation of liver microsomes[33].

Broggini *et al* detected a product of the *in vitro* enzymatic reaction obtained after horseradish peroxidase/hydrogen peroxide oxidation of VP16 by HPLC, and identified it as 1,2,3,4-tetradehydro-VP16 by ^{1}H NMR and mass spectrometric analysis[34].

STABILITY AND DEGRADATION

VP16 is extensively degraded at pH <2 and pH >8. Degradation is negligible at pH of 3.05-7.30 within 48 h[35]. VP16 is degraded with apparent first-order kinetics. At pH 1.29, the degradation half-life and rate constant are 2.85 h and 5.84 ± 0.09 day^{-1}, respectively.

From data by Holthuis and Van Oort, when the pH of the biological samples, the washing solutions and mobile phase is maintained between 4 and 9.3, solutions of VP16 and VM26 are stable for several months[36]. The *in vitro* stability and compatibility of daunorubicin, cytarabine, and VP16 have been further evaluated by Kobrinsky *et al*[37]. VP16 was found to be stable over 72 h in 5% dextrose and 0.45% saline solution either alone or combined with two other drugs. Precipitates or color changes were not noted. Changes in the patterns of the UV spectral scans and chromatographs were not observed. Concentrations of the drugs as assessed twice daily by HPLC were stable over the 72 h observation period.

In an environment-protection study, Benvenuto *et al* reported that VP16 and VM26 are immediately destroyed by potassium permanganate ($KMnO_4$) or by 5.25% sodium hypochloride (bleach). However, in comparison with the total destruction of VM26 by 1N hydrochloric acid (HCl) in 24 h, treatment of VP16 with the same conditions results in only incomplete destruction (36 % VP16 remaining)[38].

BIOANALYSIS OF VP16

In the early 1970s, C_4-tritium-labeled VP16 was used for pharmacokinetic studies of VP16 in animals and man using a radiochemical analytical assay[39]. This method is no longer used because

Table 1. Comparison of sensitivities of currently available bioanalytic methods for VP16

Methods	Sensitivity	References
HPLC with UV detection	30-100 ng/ml plasma	(36), (40)
HPLC with fluorescence detection	50 ng/ml plasma	(41)
HPLC with electrochemical detection	~2 ng/ml plasma	(20)
HPLC with mass spectrometric detection	< 1 µg/ml	(42)
RIA by Aherne *et al*	5-10 ng/ml plasma	(43)
RIA by Ho *et al*	30 ng/ml plasma	(21)
RIA by Miyazaki *et al*	1 ng/ml plasma	(44)
ELISA using Phosphatase	6 ng/ml plasma	(45)
ELISA using Horseradish peroxidase	0.5 ng/ml plasma	(46)
ELISA using ß-D-Galactosidase	40 pg/ml plasma	(47)
Flow-injection analysis (FIA)	1.5 µg/ml	(48)
Plasma desorption mass (PDMS)	unclaimed	(49)

it lacks selectivity and is not appropriate for clinical pharmacokinetic studies as it requires the administration of radioactive material to patients.

However, in the past fifteen years, many high-performance liquid chromatography (HPLC) analyses for VP16 have been established and have been extensively used in metabolic or pharmacokinetic studies. In addition to these HPLC methods, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), flow-injection analysis (FIA), and plasma desorption mass spectrometry (PDMS) have been developed and are available for the bioanalysis of VP16. The sensitivities of the various analytical methods are compared in Table 1. Among them, ELISA using β-D-galactosidase, which was developed by Takata *et al*, provides the highest sensitivity (40 pg/ml plasma).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC methods have become more and more popular in the bioanalysis of VP16. Table 2 summarizs the diverse conditions used in the analysis of VP16 and VM26 in biological fluids. Some of the references also include the HPLC analysis of possible metabolites.

Sample Preparation

In most analytical determinations, biological samples such as plasma, urine, or bile should be purified by solvent extraction, in order to remove protein, water soluble impurities, or other interfering materials. Dichloromethane and chloroform are frequently used as extraction solvents for VP16 or VM26. Littlewood *et al* has also investigated the extraction percentages of 1,2-dichloroethane, dichloromethane, chloroform, and ethyl acetate for the extraction of VP16 and VM26 from plasma[50]. The extraction percentage was greater than 90% for each solvent, but 1,2-dichloroethane produced the fewest interfering chromatographic peaks. Holthuis and Van Oort also pointed out that chloroform, dichloromethane, ethyl acetate, and 1-pentanol were suitable for extraction of VP16 and VM26, with partition coefficients larger than 25[36]. Furthermore, the organic layer could be washed twice with 0.01 M phosphate buffer (pH 7.3) to remove some of the interfering compounds. The height of several interfering peaks then decreased remarkably without changing the recoveries of VP16 and VM26. The improvement obtained by these washings was more noticeable with detection at 280 nm compared to 254 nm[36].

In lieu of solvent extraction, Strife *et al* used a small XAD-4 ion exchange resin column to separate the hydroxy acid from plasma[18].

Eisenberg *et al* reported a method for determination of VP16 in dog whole blood. The procedure includes solvent extraction of VP16 using a dichloroethane-hexane (1:1, v/v) mixture and reconstitution of the drug in an aqueous reconstitution solution[51].

Recently, sodium dodecyl sulphate (SDS) was added to the mobile phase used to load untreated plasma samples onto a precolumn. The proteins are solubilized by the SDS and washed out, whereas the analyte is retained. Next, the retained analyte and some residual endogenous compounds are eluted with the chromatographic mobile phase and analyzed using conventional HPLC. This method has been successfully applied to an automatic on-line HPLC for the plasma assay of VP16 and VM26[52].

Precolumn

Precolumns or guard columns have been used frequently in HPLC analysis since the 1980's for simplification of sample preparation, protection of analytical column or trace enrichment.

Nielen *et al* designed a miniaturized precolumn for on-line trace enrichment in narrow-bore liquid chromatography and studied critical parameters such as precolumn length, inlet capillary I.D., and flow-rate[53]. The precolumn allows direct injection of VP16-containing plasma and serum samples without any previous clean-up. Due to the trace enrichment, concentration sensi-

tivity is improved 50-fold when 100 μl samples are applied as compared to standard micro-loop injections of 0.5 μl.

Gimmel and Maurer used precolumns in their pharmacokinetic study[54]. Namely, 200μl of elute leaving the ultrafiltration-flat chamber were directly injected into a precolumn (LiChrosorb RP-18, particle size: 7μm, 25 mm x 4 mm I.D., Merck). The hydrophilic compounds were separated by rinsing with a solvent consisting of 0.1% trifluoric acid (TFA)/10% acetonitrile in HPLC grade water. VP16 was then concentrated on the precolumn.

Columns

All HPLC analyses of VP16 use reverse phase silica gel columns.

In the early 1980's, the determination of VP16 and VM26 by HPLC mostly used an ODS column for separation[18,20,41,55,56]. Strife *et al* were unable to resolve VP16 and *picro*-VP16 on C18 columns[41], but used a phenyl-bonded column for their separation[18]. As shown in Table 2, a μBondapak phenyl column has been used in most analytical determination. The *picro* isomers of VP16 and VM26 can also be readily resolved from the parent compounds by a 5μM ODS-Hypersil column with acetonitrile-0.5 M ammonium acetate buffer (pH 6) (37.5:62.5), containing 100 mg/l of EDTA as the eluent[57]. Under these conditions, the on-column detection limits for VP16 and VM26 were 500 and 750 pg injected respectively, based on a signal-to-noise ratio of 3.

One of most pronounced interferences with VP16 proved to be caffeine, which could not be removed from plasma by solvent extraction. Caffeine and VP16 show nearly the same capacity factors on C18 μBondapak reversed-phase columns. Variation of the composition of the mobile phase did not improve the separation. However, better separation was obtained with Lichrosorb columns, which have a lower surface coverage of chemically bonded C-18 groups[36].

A radial-compression module (Z-module) equipped with a 5-μm particle-size C18 packed, 8mm X 10 cm, 8NV C18 Radial Pak cartridge, has been used for assay of VP16 with electrochemical detection and resulted in high efficiency and rapid elution[58]. The peaks corresponding to both VP16 and VM26 were sharp and symmetrical. Their retention times were 5.35 and 15.75 min, respectively.

Detection Methods

Four detection methods, UV, fluorescence, electrochemistry, or time-of-flight mass spectrometric detection, have been used for the analysis of VP16 or VM26.

The first HPLC analysis of VP16 from plasma by UV 252 nm detection was reported in 1980 by Allen[55]. As shown in the Table 2, the most commonly used wavelength for VP16 detection is 254 nm. Other wavelengths including 229, 230, 233, 252, 254, 277, 280, 284, 286 and 288 nm have been employed. However, Chow and Shan examined three calibration curves of 5-30 ng/ml of VP16 with absorbance measurements at 230, 254, and 286 nm, respectively[35]. Monitoring at 230 nm yielded the greatest assay sensitivity as reflected by the highest slope value. An HPLC method described by Holthuis and Van Oort utilized UV 280 nm detection and had a limit of quantitation of 30 ng/ml[36].

The first fluorescence detection of VP16 and VM26 was mentioned in 1981 by Strife with an excitation wavelength of 215 nm and emission wavelength of 328 nm[41]. Fluorescence detection is about 10-fold more sensitive than the frequently used UV 254 nm detection. The assay can be used with confidence to 50 ng of VP16 or VM26 per ml of plasma, and presence of the drug can be confirmed at concentrations as low as 25 ng/ml plasma.

VP16 and VM26 contain phenolic groups that can be easily oxidized, therefore, an electrochemical detector is ideal for the sensitive and specific detection of these lignan compounds. They can be detected at the low ng/ml concentration ranges, which is at least 10-30 times more sensi-

tive than the UV detection. The first electrochemical detection of VP16 and VM26 from plasma was reported by Evans *et al* in 1982[19]. The electrochemical detection described by Duncan *et al* increased the sensitivity to 10 ng/ml[40]. This concentration is well below that likely to be found in the sera of patients undergoing treatment. Optimal potentials for electrochemical detection of VP16 and VM26 were +850mV and +750 mV, respectively from their cyclic voltammograms[23]. Podophyllotoxin was not oxidized, and its voltammogram was not substantially different from the solvent scan. Since the 4'-demethylepipodophyllotoxins (aglycone) of VP16 and VM26 possesses electrochemical activity, the moiety responsible for the electroactivity of VP16 and VM26 may be the phenolic hydroxy group on the E-ring of the molecules. Ploegmakers and Mertens have developed an automatic, reliable, dynamic oxidative electrochemical detection HPLC system for the on-line determination of VP16 and VM26 in effluents[59]. The detection was done at constant potential (+800 mV vs. Ag/AgCl) or by using potential scans. Ploegmakers *et al* [60] reported the implantation of software filters for improved collection and handling of electrochemical detection signals.

The only time-of-flight mass spectrometric detection of VP was reported by Danigel *et al*, and has been used in on-line drug monitoring of VP16[42]. The detection utilizes ^{252}Cf fission fragment-induced ionization and desorption of non-volatile compounds. The detection limit for VP16 is less than 1 μg/ml.

Mobile Phase

VP16 is most stable in the pH range of 5-6.15 and rapidly degrades at pH <2.03 and pH >8. The half-life time of VP16 at pH 1.30 was 2.85 h[61]. Therefore, slightly acidic mobile phases were used in most cases to prevent the possibility of base-catalyzed isomerization of VP16 or VM26 to the biological-inactive *picro* series.

A mobile phase of water-acetonitrile-acetic acid (74:25:1) at a flow-rate of 1 ml/min produces retention times for VP16 and VM26 of 13 and 20 min[19], respectively, whereas using a mobile phase of methanol-phosphate buffer (60:40), retention times of 5 and 9 min were obtained. Littlewood *et al* investigated differing proportions of methanol relative to the phosphate buffer and found that a ratio of 60:40 produced optimum chromatography with short retention times and good resolution between VP16 and VM26[50].

Internal Standard

In most cases, for the measurement of VP16 in biological fluids, VM26 has been used as an internal standard, and vice versa. However, the large capacity factor and poor efficiency of VM26 combine to give extended run times. To shorten analysis time, an assay using diphenylhydantoin (DPH) as an internal standard has been reported by Harvey and Joel[62] with retention times of 2.1 min for VP16 and 2.9 min for DPH. In some cases, 4'-demethylepipodophyllotoxin (aglycone)[63], peltatine[57], the ethyl ester of *p*-hydroxybenzoic acid[36], methoxypsoralen[35,61], phenacetin[64], and phenytoin[65], are also used as internal standards.

FLOW-INJECTION ANALYSIS (FIA)

Flow-injection analysis (FIA) and HPLC show great similarity in instrumentation and in operational parameters. The main differences are the use of a reactor in FIA and a separation column in HPLC. An on-line FIA analysis for determination of VP16 and VM26 in plasma has been established by Van Opstal *et al*[79]. A dual-electrode detector with a potentiostat was used as the electrochemical reactor in place of a column in the assay. It consists of a low volume (5μl) flow-through cell, which contains two porous graphite working electrodes. Oxidation potentials of +500mV and +450mV for VP16 and VM26, respectively, were selected. The 365 nm band caused by the oxidation was selected as the detec-

Table 2. HPLC analysis conditions for VP16 and VM26 and their metabolites

Column	Mobile phase	Detection	Reference
μBondapak phenyl 10μm	CH_3CN-H_2O-AcOH(74:25:1), 1.0 ml/min; 0.02 M NaOAc Buffer (pH 4.0)+26% (or 60%) CH_3CN; MeOH-H_2O-CH_3CN (50:45:5)+10mM ammonium acetate (pH 5.5), 2.0 ml/min; MeOH-H_2O (60:40), 1.0 ml/min; CH_3CN-0.01M sodium acetate (adjusted to pH 3.8 with AcOH), (30:70), 1.0 ml/min; MeOH-10 mM sodium phosphate buffer (pH 7) (55:45), 1.0 ml/min; H_2O-CH_3CN-AcOH(62:37:1), 1.5 ml/min; H_2O-CH_3CN-AcOH(64:35:1), 1.0 ml/min	UV 254 nm, UV 230 nm, UV 277 nm, UV 284 nm, UV 285 nm	(18),(25), (34),(66), (67),(14), (33),(52), (68),(69), (23),(22)
μBondapak phenyl, 5 μ m	H_2O-CH_3CN-AcOH(70:30:1), 1.0 ml/min	UV 233 nm	(70),(71)
μBondapak C18, 10 μ m	MeOH-H_2O (50:50), 1.5 ml/min	UV 254 nM	(38)
LiChrosorb RP-18, 5 μ m	MeOH-H_2O (55:45), 1.0 ml/min; MeOH-H_2O-AcOH (50:49:1, pH 3.33), 1.0 ml/min; MeOH-H_2O-AcOH (45:54:1, pH 3.28), 1.0 ml/min; gradient: A: H_2O-TFA (999:1, pH 2)+B: H_2O-CH_3CN-TFA (399:600:1)	UV 254 nm, UV 280 nm	(56),(36), (54)
Radial-pak C_{18} 10 μ m	MeOH-CH_3CN-H_2O (2:1:1), 1.0 ml/min	UV 254 nm	(42)
reverse-phase C8	CH_3CN-AcOH-H_2O (27:1:72), pH 4.0, 1.0 ml/min	UV 230 nm UV 254 nm UV 286 nm	(61),(35)
MCH-10 Micropak (C_{18} reverse)	A:Na_3PO_4 buffer (10 mmol/L)-3% CH_3CN, B:100% CH_3CN, gradient from A(100%)/B(0%) to A(45%)/B(55%)	UV 235 nm; UV 254 nm	(37)
Microsphere C18, 3μm	MeOH-0.05 M potassium phosphate buffer (pH4) (40:60), 0.5 ml/min	UV 280 nm	(72)
ODS-Hypersil, 5μM	MeOH-H_2O (51:49), 1.5 or 2 ml/min	UV 229 nm	(62),(65)
Capcelapk C18, SG120	potassium phosphate buffer-CH_3CN (20 mM, pH 4.5) (68:32), 0.5 ml/min	UV 288 nm	(73)

Table 2. Continued

Partisil-10 ODS-2	CH_3CN-H_2O (28:72), 1.0 ml/min	UV 252 nm	(74)
μBondapak C18, 10 μ m	MeOH-H_2O (60:40), 1.0 ml/min	fluorescent (215/328 nm)	(41)
LiChrosorb RP-18, 10μm	H_2O-MeOH-CH_3CN-AcOH (50:40:8:2), 1.0 ml/min; MeOH-H_2O (55:45), 1.0 ml/min;	fluorescent: 288/328 nm; 230/328 nm	(75),(63)
LiChrosorb RP-18, 5μm with precolumn	Pumb A:40% CH_3CN, 100 μl/min + Pump B: H_2O, 200 μl/min	fluorescent: 230/328 nm	(53)
Nucleosil C18, 5μm	MeOH-H_2O-AcOH (46:64:1, pH 3.3), 1.0 ml/min	fluorescent: 230/328 nm	(63)
Econosil C18, 10μm	MeOH-CH_3CN-H_2O-AcOH (39:15:45:1, pH 3.0), 1.0 ml/min;	fluorescent. 230/328 nm	(76)
μBondapak phenyl, 10μm	H_2O-CH_3CN-AcOH (74:25:1), 1.0 ml/min; MeOH-10 mM sodium phosphate buffer (pH 7) (55:45), 1.0 ml/min; MeOH-0.065 M phosphate buffer (pH 7) (40:60), 1.0 ml/min; H_2O-CH_3CN-AcOH (68:30:2), 1.0 ml/min	*ECD: 850mV	(23),(19), (20),(77), (52),(51)
μBondapak phenyl, 5μm	MeOH-phosphate buffer (60:40), 1.0 ml/min	ECD: + 800mV	(50)
LiChrosorb RP-18	MeOH-0.020 M Phosphate buffer (pH 7) (55:45), 1.0 ml/min	ECD	(59),(60)
μBondapak CN	CH_3CN-0.02M sodium acetate (pH 4.0), (17.7:82.3), 1.8 ml/min; CH_3OH-0.03M sodium acetate containing 0.1 g/l EDTA (pH 4.0), (4:7), 1.0 ml/min	ECD:700mV	(40),(78)
8NV C_{18} Radical Pak, 5μm	MeOH-10mmol/L sodium monobasic phosphate (adjusted to pH 3 with 85% phosphoric acid), 2 ml/min	ECD	(58)
ODS-Hypersil, 5μM	CH_3CH-0.5M ammonium acetate buffer (pH 6) (37.5:62.5) + 100 mg/l EDTA, 1.0 ml/min	ECD: + 900mV	(57)
Novapak phenyl, 4μm	MeOH-phosphate buffer, pH 7 (55:45), 1.0 ml/min	ECD:500mV	(48)

*ECD:Electrochemical detection.

tion wavelength. Van Opstal and Krabbenborg further compared a flow-injection method with an HPLC method for the assay of VP16 in plasma[48]. The results were evaluated statistically, providing specific estimates of the type and magnitude of errors. Both FIA and HPLC showed good linearity in all ranges. The correlation coefficients were at least 0.999 for all curves. The mean variances (S.D.^{2}) for FIA and HPLC were calculated and proved to be 1.164 and 0.574 (μg/ml)^2, respectively, in the investigated concentration range of 1-25 μg/ml. FIA of VP16 in plasma is a good alternative to HPLC analysis, when plasma levels are at least 1.5 μg/ml. The rapid stabilization, the flexibility, and the high speed of analysis makes FIA attractive for routine control analysis of patient plasma.

RADIOIMMUNOASSAY

The first radioimmunoassay for pharmacokinetic studies of VP16 was developed by Aherne *et al*[43]. A ^{3}H-VP16 bovine serum albumin (BSA) conjugate was prepared using the sodium periodate reaction and was injected into three sheep to produce antisera. Histamine was conjugated to ^{3}H-VP16 using the same sodium periodate reaction, was further radioiodinated using Iodogen, and then was used as the marker (or iodinated label). The precise chemical nature of the conjugate is unknown. Since podophyllotoxin does not cross-react with the antiserum used in the assay, and VM26 cross-reacts only to a small extent (< 0.6%), it can be assumed that the main antigenic determinant is the ethylidene glucosidic moiety of VP16. VP16 can be measured in plasma without prior drug extraction with a theoretical limit of detection of 5-10 μg/l. However, it was found that the antiserum recognized the *picro*-hydroxy acid analogue more than VP16 itself. This may be attributable to the conversion of VP16 to *picro*-VP16 or *picro*-hydroxy acid analogues under the alkaline conditions of the coupling process. Thus, in order to eliminate any interference from the *trans*-hydroxy acid metabolite, chloroform extraction of plasma samples was carried out.

Subsequently, Ho *et al* developed a reproducible and sensitive radioimmunoassay for VP16 or VM26 (Figure 3)[21]. The antiserum was produced by immunizing rabbits with succinyl-VP16-BSA conjugates. To synthesize succinyl-

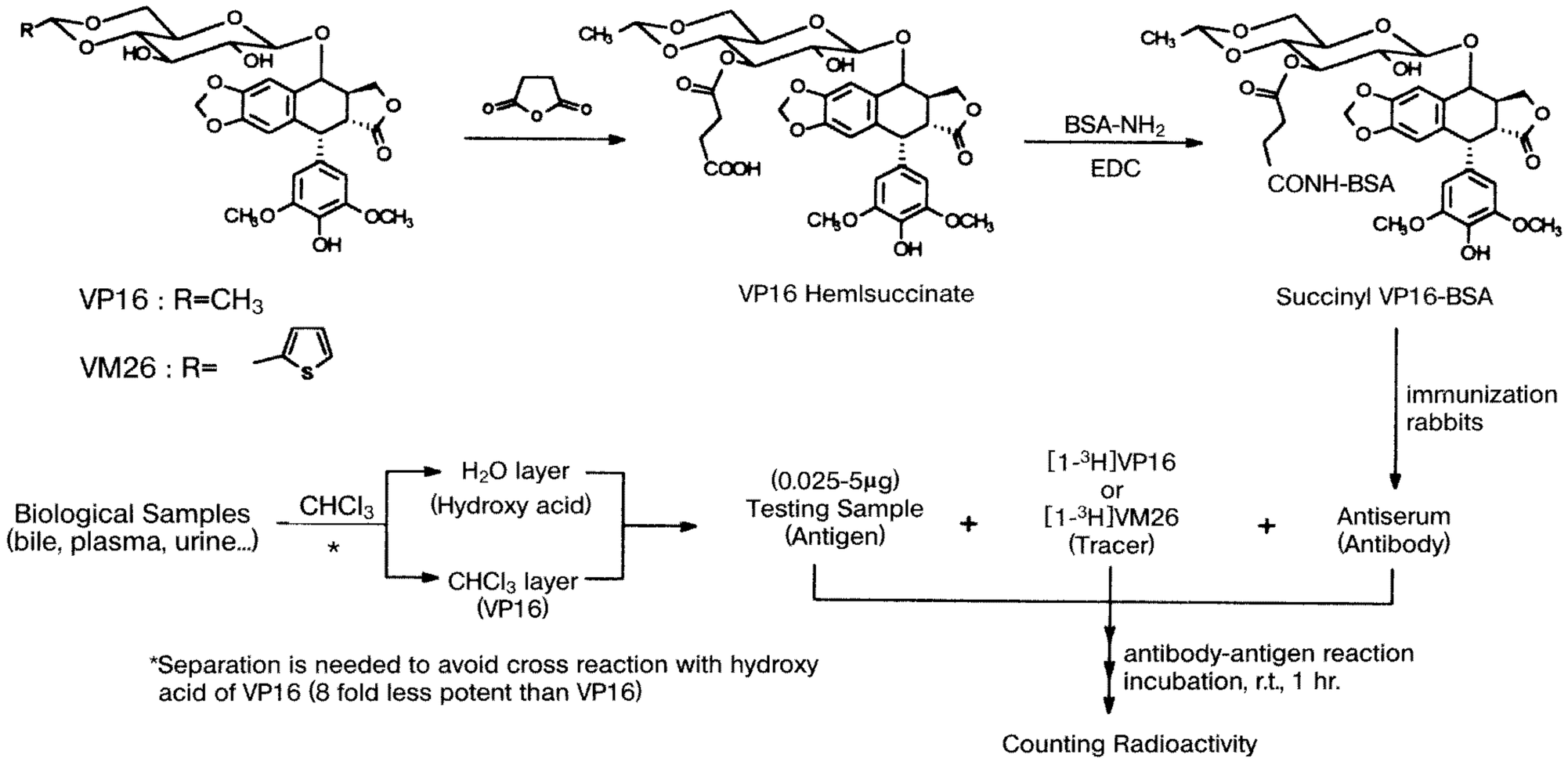


Figure 3. Aradioimmunoassay for the determination of VP16 in human plasma.

VP16, carboxyl groups were first introduced into the VP16 molecule by converting its hydroxy group to the half ester of succinic acid. This enol hemisuccinate was very unstable and, therefore, was coupled to BSA without purification. Using the trinitrobenzene sulfonic acid method for the determination of the primary amine, the conjugate was estimated to contain 25 succinyl-VP16 residues per BSA molecule. However, the hydroxy acid of VP16 cross-reacted with the VP16 antiserum and achieved 50% inhibition of binding at approximately 0.55 μg/assay, which is approximately 8-fold less potent than VP16. However, VP16 can be easily extracted into chloroform and separated from the non-extracted hydroxy acid. The alternate ligand, tritiated VM26, also reacted with the VP16 antisera and demonstrated identical sensitivity to that of tritiated VP16. Thus, this assay can be used for measuring both drugs. The assay was reproducible and sensitive. Extracted standard curves were linear from 0.025 to 5 μg for VP16 and 0.1 to 10 μg for the hydroxy acid per 0.5 ml assay mixture.

Consequently, Miyazaki *et al* developed an alternative radioimmunoassay for the determination of VP16 in human plasma[44]. VP16-acetonyl-carboxymethoxime-BSA and VP16-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 VP16 from *picro*-VP16 and *picro*-hydroxy acid. Good correlation was found in a comparison of the new RIA method with an HPLC method (r = 0.9792). The method was applied to the direct analysis of VP16 in plasma obtained from cancer patients treated intravenously (200 mg man^{-1}) and orally (400 mg man^{-1}).

ENZYME-LINKED IMMUNOSORBENT ASSAY

The first enzyme-linked immunosorbent assay (ELISA) for VP16 was developed by Hacker *et al*[45]. A brief description of the assay is as follows. VP16-antibody and alkaline phosphatase conjugate (VP-E) were produced by a $NaIO_4$:$NaBH_4$ procedure. VP16-antibody was diluted in $NaHCO_3$ buffer (pH 9.0), and 100 μl aliquoted to each well in a 96 well microtiter plate. After 18 h at 4°C, the wells were washed with phosphate buffered saline-Tween 80 (PBST), and 50 μl of a known concentration of VP16 and 50 μl of VP-E were added to the appropriate wells. The wells were washed with PBST 18 h later, and 100 μl of *p*-nitrophenylphosphate was added to each well. The amount of enzyme present is directly proportional to the increase in absorbance at 405 nm. Since free VP16 and VP-E compete for the VP16-antibody, the amount of enzyme present is inversely proportional to the amount of free VP16 added to the well. The ELISA assay is sensitive to VP16 levels as low as 6 ng/ml.

An ELISA developed by Henneberry *et al* [46] for determining VP16 in unextracted plasma utilized VP16-thyroglobulin-coated microtitre plates, a sheep anti-VP16 serum, and a donkey anti-sheep horseradish peroxidase (HRPO)-labelled second antibody. The sensitivity of the assay was 0.5 ng/ml plasma. A good correlation was obtained between the ELISA and established HPLC (r = 0.97) and RIA (r = 0.96) methods. The antiserum did not cross-react with metabolites of VP16.

Saita *et al* developed a more sensitive ELISA utilizing β-D-galactosidase instead of alkaline phosphatase[47]. The procedure is shown in Figure 4. Anti-VP16 sera were produced by immunizing rabbits with VP16 conjugated with mercaptosuccinyl bovine serum albumin (VP16-BSA) using N-[β-(4-diazophenyl)ethyl]maleimide (DPEM) as a heterobifunctional coupling agent. An enzyme marker was similarity prepared by couplingVP16 with β-D-galactosidase (β-Gal; EC 3.2.1.23) *via* DPEM. The azo-coupling of VP16 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 VP16-DPEM was

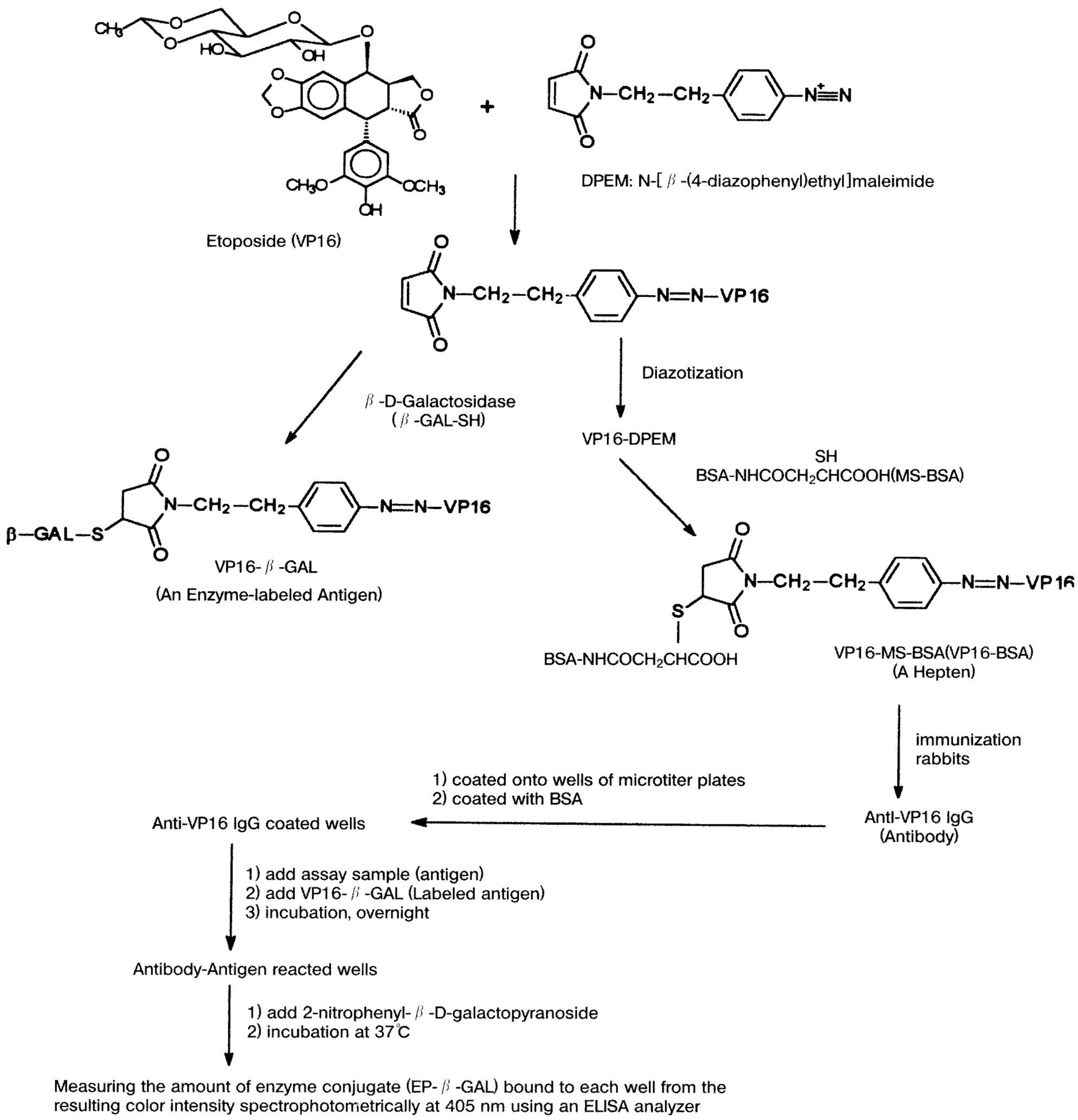


Figure 4. A highly sensitive enzyme-linked immunosorbent assay (ELISA) for VP16.

conjugated by thiolation with the thiol groups of MS.BSA (immunogen) and β -Gal (enzyme marker). This ELISA was specific for VP16 and showed a very slight cross-reactivity with its major metabolite, *cis*-hydroxy acid of VP16 (0.91%), a major metabolite seen in plasma and urine, and no cross-reactivity with 4'-demethylepipodophyllotoxin, the aglycone moiety of the parent drug. This suggests that the antibody-recognition site is at the sugar moiety and at the lactone ring of the aglycone moiety of the VP16 molecule. This degree of cross-reactivity is much better than the high cross-reactivity with the *cis*-hydroxy acid of VP16 shown in the radioimmunoassay[21,43]. Anti-VP16 antibody showed no cross-reaction with various other anticancer drugs commonly used with VP16 in combination cancer chemotherapy, such as adriamycin, cyclophosphamide, methotrexate, mitomycin C, and vincristine. The values for VP16

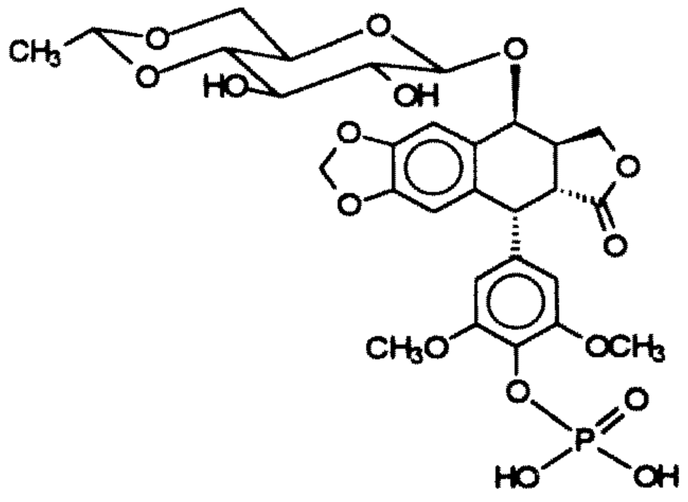
concentration detected by this assay were comparable with those determined by the HPLC method. However, the ELISA was about 1,250 times more sensitive in detecting VP16 at lower concentration. Using this assay, drug levels were easily determined in the blood and urine of rats for 7 h after *i.v.* administration of VP16 at a single dose of 3 mg/kg. An established optimal VP16 ELISA assay procedure is capable of accurately measuring as little as 40 pg VP16/ml. This sensitivity appears to be 1,250 and 125 times more sensitive than the present HPLC procedure and the previous radioimmunoassay[43] for VP16, respectively.

PLASMA DESORPTION MASS SPECTROMETRY (PDMS) ANALYSIS

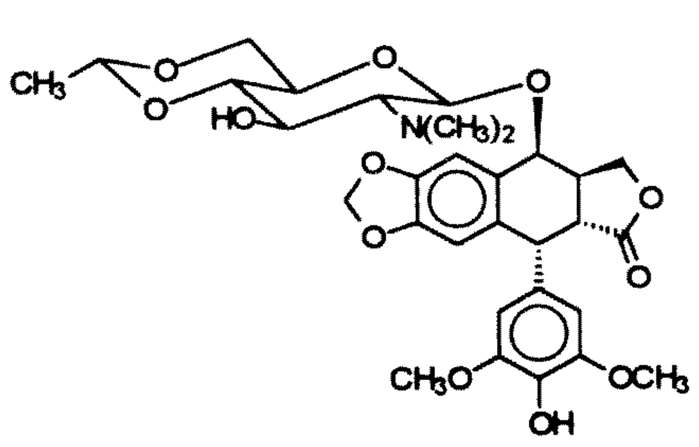
^{25}Cf-Plasma Desorption Mass Spectrometry (PDMS) is usually used in structural analysis and for the determination of molecular weights. However, in VP16 analysis, quantitative matrix assisted PDMS has proven to be an effective and reliable tool. Applying this tool, the pharmacokinetics of standard-dose VP16 (70-150 mg/m^2) for the treatment of several malignancies have been investigated extensively[80]. In another study, 27 high-dose kinetics of ten patients were analyzed[81]. Recently, Jungclas *et al* performed an improved quantitative matrix assisted PDMS analysis for the pharmacokinetic studies of VP16[49]. However, in extracted serum and urine samples, salts as well as organic impurities are still present in amounts which usually cannot be tolerated in PDMS. Purification of sample on the PDMS-target could be archived by liquid chromatography. Plasma samples containing VP16 are labeled with VM26 as internal standard and analyzed in a four-step procedure: (i) sample extraction, (ii) sample purification by TLC, (iii) target preparation, and (iv) quantitative PDMS detection. The quantitative results are obtained by comparing the two mass lines corresponding to the molecular ions of VP16 and VM26. Practically, the sample, approximate 10 μg, was embedded in a matrix of 100 μg sucrose octaacetate and 150 μg urotropin in order to: (1) reduce the ion intensities related to impurities, (2) promote the release of molecular ions by protecting the sample molecules against the chemical attack of impurities in the desorption process, and (3) improve the mixture homogeneity of the two drugs in the prepared target.

NEW ANALOGS OF VP16

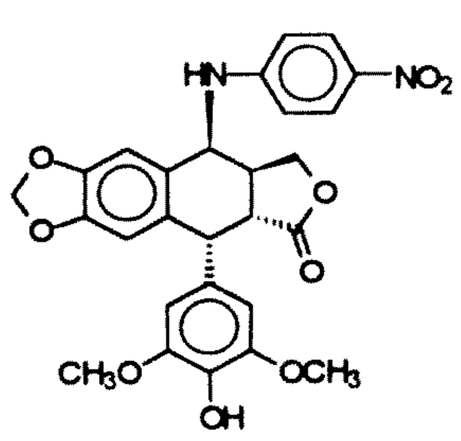
Due to problems with VP16 or VM26 such as toxicity, poor water solubility, and the development of tumor cells resistance, investigators are still exploring new analogs for a new lead compound with an enhanced therapeutic index, an extensive therapeutic scope and higher water solubility. Hundreds of new derivatives have been synthesized and biologically evaluated. These have been summarized in our recent review[13]. From these endeavors, three analogs, etoposide phosphate, NK-611, and GL331, have entered clinical trials.



Etoposide Phosphate



NK-611



GL331

Etoposide Phosphate (BMY-40481)

This is a new water-soluble analog of VP16, which acts as a prodrug, probably through activation by plasma phosphatase. *In vitro*, VP16 phosphate was less potent than VP16; however, in animals, VP16 phosphate is converted to VP16 within a few minutes, regardless of mode of administration[82]. The phase I clinical and pharmacokinetic study of oral etoposide phosphate has been reported by Sessa *et al*[69].

NK611

NK611, 4'-demethylepipodophyllotoxin-9(2-deoxy-2-dimethylamino-4,6-O-ethylidene-β-D-glucopyranoside) developed by Nippon Kayaku (Tokyo, Japan), is a new water-soluble derivative of VP16 with potent antineoplastic activity[73]. A dimethylamino group was introduced into the sugar moiety to make the water-soluble amine salt. The simultaneous determination of NK611 and its metabolite (DeNK611) in dog plasma by column-switching HPLC has been reported by Machida and Tanaka[73]. The clinical pharmacokinetic studies of NK611 have been investigated by D'Incalci *et al*[83].

GL331[84]

Recently, GL331, a new derivative of epipodophyllotoxin developed in our laboratory and licensed to Genelabs, Inc., California, has been brought into phase I clinical study in the United States. GL331, a novel topoisomerase II inhibitor of the epipodophyllotoxin family, has been evaluated in a variety of *in vitro* and *in vivo* systems, and has shown activity in both VP16 sensitive and resistant cell lines and in animal models. GL331 shares many properties with VP16. The results indicated that, for DNA topoisomerse II, the concentrations of GL331 and VP16 required to inhibit the unknotting activity (ID_{50}) were 10 and 50 μM, respectively. GL331 demonstrated a cytotoxicity (IC_{50}) in KB cells (human nasopharyngeal carcinoma) comparable to VP16, with cellular protein-DNA complex formation approximately three times that of VP16. The cytotoxicity study of GL331 on parental and various multi-drug resistance (MDR) cell lines indicated that GL331 was consistently more cytotoxic than VP16 against six parental human and murine tumor cell lines. Similar results were observed in twelve well-characterized MDR variants. Compared with VP16, GL331 caused two to three times more protein-linked DNA breaks in the nasopharyngeal carcinoma (KB) cell line and ten times more in the KB/vcr cell line. The concentrations of GL331 required to achieve IC_{50} in the non-resistant cell lines were comparable to those of VP16. However, GL331 was approximately 40 times more potent than VP16 in adriamycin-resistant P388 murine lymphoma cells. To better understand why GL331 is more effective than VP16 in MDR cell lines, the uptake of radiolabelled GL331 and VP16 was examined in a panel of both parental and MDR cell lines. Results indicated that GL331 uptake was significantly higher than VP16 in both parental and MDR variants. In addition, the efflux of radioactive GL331 and VP16 was assessed in parental and vincristine-resistant KB cell lines. GL331 and VP16 were pumped out of KB cells at a similar rate. However, the efflux of VP16 in vincristine-resistant cells was significantly higher than that of GL331. The cytotoxicity kinetics of GL331 and VP16 indicated that GL331 achieved the same degree of cell killing as VP16 in approximately half the time in parental KB cells. In vincristine-resistant KB cells, GL331 achieved comparable levels of cytotoxicity in approximately one-sixth the time of VP16. It was concluded that the relatively superior antitumor activity of GL331 compared with VP16 was probably due to better drug transport across both parental and MDR cells.

The selectivity and potency of GL331 against tumors were evaluated by the National Cancer Institute (NCI) using the in vitro screening of drug cytotoxicity against a human tumor panel consisting of sixty tumor cell lines derived from nine histological types. Results from two

experiments indicated that leukemia, non-small cell lung cancer, colon cancer and renal cancer exhibited preferential susceptibility to GL331. The in vitro effects of GL331 and VP16 on the cell cycle progression in both parental cells and a drug resistant variant were analyzed by counting the population of treated cells at different phases of the cell cycle. After exposure to either GL331 or VP16 at their corresponding IC_{50}, KB cells were largely arrested in G2/M phase. If the cells were treated by either drug at higher concentrations, S phase was also arrested with a decreased G2/M blockage. In BDF_1 mice inoculated intraperitoneally (i.p.) with P388 lymphoma, GL331 demonstrated greater increase in life span (ILS) for the GL331 treated mice (30 mg/kg) as compared to VP16. GL331 administered intravenously showed an ability to increase the lifespan (ILS) of BDF_1 mice bearing adriamycin-resistant P388 lymphoma cells. In another murine leukemia tumor model, mice bearing either parental (L1210/0) or drug-resistant variant (L1210/adr) were treated with GL331 and VP16 at the same regimens and dosages. GL331 achieved optimal therapeutic effect against L1210/0 (GL331, 13.5 mg/kg) in terms of ILS, long-term survival, and tumor burden reduction at approximately half the dosage of VP16. L1210/adr (GL331, 13.5 mg/kg) *in vivo* appeared to be less responsive to GL331 and VP16, as expected. However, GL331 again outperformed VP16 in terms of ILS, long-term survival, and tumor burden. In two cancer models, characterized by slow growth with presumably high levels of P-glycoprotein, GL331 demonstrated significantly better antitumor effects than VP16. In female $B6C3F_1$ mice bearing colon 38 tumors implanted intramuscularly, GL331 administered i.p. resulted in tumor regression in 30 to 50% of treated animals. On day 35, 20% of the mice were tumor-free, and tumors that did not regress exhibited a significant growth delay. VP16 administration resulted in significantly less tumor regression and growth delay in mice bearing colon 38 implants. In athymic mice bearing human colorectal carcinoma xenografts, i.p. administration of GL331 at 20 mg/kg/day for 5 days delayed tumor growth, while VP16 had no effect on the xenograft after i.p. administration at the same dose and regimen. GL331 at high doses also exhibited some antitumor activity in Lewis lung carcinoma but was devoid of activity in B16 melanoma. VP16 administered to mice at the same dose and regimen was ineffective in either solid tumor model. Based on the above *in vitro* and *in vivo* antitumor data, it was concluded that the potential advantages of GL331 include its ability to overcome tumor resistance to chemotherapy, which may result in improved outcome when used as first line therapy, as well as increased salvage of patients who have failed other first line therapeutic regimens resulting from development of MDR.

The toxicological profile of GL331 was evaluated *in vivo* in rats and dogs. In rats, the estimated LD_{50} values of GL331 and VP16 following a single 2 hour i.v. infusion were 68 and 122 mg/kg, respectively; the respective LD_{10} values of GL331 and VP16 were 46 and 89 mg/kg. The target organs in the rat were testes and bone marrow when GL331 was administered for 5 days. In the dog, transient, occasionally marked elevations of liver enzymes and decreases in circulating white cell and reticulocyte counts were observed. Plasma elimination half-lives in the rat following a single 2 hour i.v. infusion were 3.51 and 1.96 hours for GL331 and VP16, respectively. Plasma elimination curves in the dog were generally similar in shape to those in the rat, both after a single 2 hour intravenous infusion and after 5 days of drug administration. Following a single 2-hour i.v. infusion of radiolabelled GL331, low levels of radioactivity were detected in plasma, liver, urine and feces in a distribution and excretion study in the rat. In addition, a very high portion of the GL331 dose was found to be bound to plasma proteins in the rat, dog, and human.

GL331's phase I trial was successfully concluded; the details of which will be published in due course.

CONCLUSIONS AND PROSPECTS

Etoposide and teniposide are highly effective and commonly used agents for both the curative and palliative treatment of a number of human malignancies. However, the mechanism of action of these compounds is not explicated completely. The oxidative activation of these compounds, which is observed *in vitro*, is probably important for the oncolytic effect *in vivo*.

For the bioanalysis of VP16 and VM26 and their metabolites, a number of HPLC methods employing UV, fluorescence, or electrochemical detection are available. Most of the HPLC methods are sensitive and reliable enough for determining the parent drugs and their metabolites in biological fluids. In addition to radioimmunoassay, an enzyme-linked immunosorbent assay (ELISA) for VP16, which is the most sensitive assay method to date, has been developed based on modern immunoassay technology.

We can expect that more detailed investigations and progress on the above new analogs will be made in the near future as well as on VP16 itself. VP16 and its analogs will remain a seminal topic in the field of cancer chemotherapy.

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REFERENCES

1. Antitumor Agents. 165. for 164 See Cho, S.J., Kashiwada, Y., Bastow, K.F., Cheng, Y.C., and Lee, K.H. 1995. Antitumor Agents. 164. Podophenazine, 2" , 3" -Dichloropodophenazine, Benzopodophenazine, and Their 4 β -para-Nitroaniline Derivatives as Novel DNA Topoisomerase II Inhibitors, J. Med. Chem. Submitted.
2. Jardine, I. 1980. Podophyllotoxins, in Cassady, J. M. and Douros, J. D. eds. "Anticancer Agents Based on Natural Product Models" , Academic Press, Chap. 9, pp. 310-347.
3. Issell, B.F. 1982. The Podophyllotoxin Derivatives VP16-213 and VM26, Cancer Chemother. Pharmacol. 7:73-80.
4. Cavalli, F. 1982. VP16-213 (Etoposide), a Critical Review of Its Activity, Cancer Chemother. Pharmacol. 7:81-85.
5. Creaven, P.J. 1982. The Clinical Pharmacology of VM26 and VP16-213, a Brief Overview, Cancer Chemother. Pharmacol. 7:133-140.
6. O'Dwyer, P.J., Leyland-Jones, B., Alonso, M.T., Masoni, S., and Wittes, R.E. 1985. Drug Therapy, Etoposide (VP-16-213), Current Status of an Active Anticancer Drug, N. Engl. J. Med. 312:692-700.
7. Van Maanen, J.M.S., Retel, J., De Vries, J., and Pinedo, H.M. 1988. Mechanism of Action of Antitumor Drug, Etoposide: a Review, J. Nat. Can. Inst. 80:1526-1533.
8. Holthuis, J.J.M. 1988. Etoposide and Teniposide. Bioanalysis, Metabolism and Clinical Pharmacokinetics, Pharm. Weekbl. 10:101-116.
9. Fleming, R.A., Miller, A.A., and Stewart, C.F. 1989. Etoposide: an Update, Drug Reviews 8:274-293.
10. Stähelin, H.F. and Von Wartburg, A. 1989. From Podophyllotoxin Glucoside to Etoposide, Prog. Drug. Res, 33:168-266.
11. Stähelin, H.F. and Von Wartburg, A. 1991. The Chemical and Biological Route from Podophyllotoxin Glucoside to Etoposide: Ninth Cain Memorial Award Lecture, Cancer Res. 51:5-15.
12. Belani, C.P., Doyle, L.A., and Aisner, J. 1994. Etoposide: Current Status and Future Perspectives in the Management of Malignant Neoplasms, Cancer Chemother. Pharmacol. 34:118-126.
13. Zhang, Y.L. and Lee, K.H. 1994. Recent Progress in the Development of Novel

Antitumor Etoposide Analogs, Chin. Pharm. J. 46:319-369.

14. Haim, N., Roman, J., Nemec, J., and Sinha, B.K. 1986. Peroxidative Free Radical Formation and O-demethylation of Etoposide(VP-16) and Teniposide(VM-26), Biochem. Biophys. Res. Commun. 135:215-220.
15. Mans, D.R., Lafleur, M.V., Westmijze, E.J., Horn, I.R., Bets, D., Schuurhuis, G.J., Lankelma, J., and Retel, J. 1992. Reactions of Glutathione with the Catechol, the Ortho-Quinone and the Semi-quinone Free Radical of Etoposide. Consequences for DNA Inactivation, Biochem. Pharmacol. 43:1761-1768.
16. Sakurai, H., Miki, T., Imakura, Y., Shibuya, M., and Lee, K.H. 1991. Metal- and Photo-induced Cleavage of DNA by Podophy-llotoxin, Etoposide, and Their Related Compounds, Mol. Pharmacol. 40:965-973.
17. Allen, L.M., Marcks, C., and Creaven, P.J. 1976. 4' -Demethylepipodophyllic Acid-9-(4,6-O-Ethylidene-b-d-d-glucopyranoside), the Major Urinary Metabolite of VP16-213 in Man, Proc. Am. Assoc. Can. Res. 17:6.
18. Strife, R.J. and Jardine, I. 1980. Analysis of the Anticancer Drugs VP 16-213 and VM 26 and Their Metabolites by High-performance Liquid Chromatography, J. Chromatogr. 182:211-220.
19. Evans, W.E., Sinkule, J.A., Crom, W.R., Dow, L., Look, A.T., and Rivera, G. 1982. Pharmacokinetics of Teniposide (VM 26) and Eetoposide (VP16-213) in Children with Cancer, Cancer Chemother. Pharmacol. 7:147-150.
20. Holthuis, J.J.M., Romkens, F.M.G.M., Pinedo, H.M., and Van Oort, W.J. 1983. Plasma Assay for the Antineoplastic Agent VP 16-213 (Etoposide) Using High-performance Liquid Chromatography with Electrochemical Detection, J. Pharm. Biomed. Anal. 1:89-97.
21. Ho, D.H., Kanellopoulos, K.S., Brown, N.S., Issell, B.F., and Bodey, G.P. 1985. Radioimmunoassay for Etoposide and Teniposide, J. Immunol. Methods. 85:5-15.
22. Dow, L., Sinkule, J.A., Look, A.T., Horvath, A., and Evans, W.E. 1983. Comparative Cytotoxic and Cytokinetic Effects of the Epipodophyllotoxins 4'-Demethylepipodo-phyllotoxin-9-(4,6-O-Ethylidene-b-d-glu-copyranoside) and 4'-Demethylepipodo-phyl-lotoxin-9-(4,6-O-2-thenylidene-b-d-glucopy-ranoside) and Their Metabolites on Human Leukemic Lymphoblasts, Cancer Res. 43:5699-5706.
23. Sinkule, J.A. and Evans, W.E. 1984. High-performance Liquid Chromatographic Analysis of the Semisynthetic Epipodophyllo-toxins Teniposide and Etoposide Using Electrochemical Detection. J. Pharm. Sci. 73:164-168.
24. Postmus, P.E., Holthuis, J.J.M., and Haaxma-Reiche, H. 1984. Penetration of VP 16-213 into Cerebrospinal Fluid after High Dose Intravenous Administration, J. Clin. Oncol. 2:215-220.
25. Colombo, T., D' Incalci, M., Donelli, M.G., Bartosek, I., Benfenati, E., Farina, P., and Guaitani, A. 1985. Metabolic Studies of a Podophyllotoxin Derivative (VP16) in the Isolated Perfused Liver, Xenobiotica 15:343-350.
26. Van Maanen, J.M.S., Van Oort, W.J., and Pinedo, H.M. 1982. in Vitro and in Vivo Metabolism of VP-16-213 in the Rat, Eur. J. Cancer Clin. Oncol. 18:885-890.
27. Van Maanen, J.M.S., Holthuis, J.J.M., Gobas, F., De Vries, J., van Oort, W.J., Emmelot, P., and Pinedo, H.M. 1983. Role of Bioactivation in Covalent Binding of VP-16 to Rat Liver and HeLa Cell Microsomal Proteins, Proc. Am. Assoc. Can. Res. 24:319.
28. Gouyette, A., Deniel, A., Pico, J.L., Droz, J.P., Baume, D., Ostronoff, M., LeBail, N., and Hayat, M. 1987. Clinical Pharmacology of High-dose Etoposide Associated with Cisplatin. Pharmacokinetic and Metabolic Studies, Eur. J. Cancer Clin. Oncol. 23:1627-1632.

29. D'Incalci, M., Sessa, C., Rossi, C., Roviaro, G., and Mangioni, C. 1985. Pharmacokinetics of Etoposide in the Gestochoriocarcinoma, Cancer Traet. Rep. 69:69-72.
30. Holthuis, J.J.M. 1985. Bioanalysis, Electrochemistry and Pharmacokinetics of Etoposide, Teniposide and Metabolites, Ph.D. Thesis. Utrecht, The Netherlands: Rijksuniversiteit Utrecht.
31. Hande, K., Anthony, T., Hamilton, R., Bennett, R., Sweetman, B., and Branch, R. 1988. Identification of Etoposide Glucuronide as a Major Metabolite of Etoposide in the Rat and Rabbit, Cancer Res. 48:1829-1834.
32. Rossi, C., Zucchetti, M., Sessa, C., Urso, R., Mangioni, C., and D' Incalci, M. 1984. Pharmacokinetic Study of VM26 Given as a Prolonged iv Infusion to Ovarian Cancer Patients, Cancer Chemother. Pharmacol. 13:211-214.
33. Haim, N., Nemec, J., Roman, J., and Sinha, B.K. 1987. In vitro Metabolism of Etoposide (VP-16-213) by Liver Microsomes and Irreversible Binding of Reactive Intermediates to Microsomal Proteins, Biochem. Pharmacol. 36:527-536.
34. Broggini, M., Rossi, C., Benfenati, E., DIncalci, M., Fanelli, R., and Gariboldi, P. 1985. Horseradish Peroxidase/Hydrogen Peroxide-catalyzed Oxidation of VP16-213. Identification of a New Metabolite, Chem. Biol. Interact. 55:215-224.
35. Chow, D., Shah, J., and Chen, J.R. 1987. Stability-indicating High-performance Liquid Chromatography of Etoposide at Various pH Conditions Using a Reversed-phase Octyl Column, J. Chromatogr. 396:217-223.
36. Holthuis, J.J.M. and Van Oort, W.J. 1981. A Sensitive High-performance Liquid Chromatographic Method for Determination of the Anti-neoplastic Agents VP 16-213 and VM 26 in Biological Fluids, Anal. Chim. Acta 130:23-30.
37. Seargeant, L.E., Kobrinsky, N.L., Sus, C.J., and Nazeravich, D.R. 1987. In vitro Stability and Compatibility of Daunorubicin, Cytarabine, and Etoposide, Cancer Treat Rep. 71:1189-1192.
38. Benvenuto, J.A., Connor, T.H., Monteith, D.K., Laidlaw, J.L., Adams, S.C., Matney, T.S., and Theiss, J.C. 1993. Degradation and Inactivation of Antitumor Drugs, J. Pharm. Sci. 82:988-991.
39. Allen, L.M. and Greaven, P.J. 1975. Comparison of the Human Pharmacokinetics of VM26 and VP16, Two Antineoplastic Epipodophyllotoxin Glucopyranoside Derivatives, Eur. J. Cancer 11:697-707.
40. Duncan, G.F., Farmen, R.H., Movahhed, H.S., and Pittman, K.A. 1986. High-performance Liquid Chromatographic Method for the Determination of Etoposide in Plasma Using Electrochemical Detection, J. Chromatogr. 380:357-365.
41. Strife, R.J. and Jardine, I. 1981. Analysis of the Anticancer Drugs Etoposide (VP 16-213) and Teniposide (VM 26) by High-performance Liquid Chromatography with Fluorescence Detection. J. Chromatogr. 224:168-174.
42. Danigel, H., Pfluger, K.H., Jungclas, H., Schmidt, L., and Dellbrugge, J. 1985. Drug Monitoring of Etoposide (VP16-213). I. A Combined Method of Liquid Chromatography and Mass Spectrometry, Cancer Chemother. Pharmacol. 15:121-124.
43. Aherne, G.W. and Marks, V. 1982. A Radioimmunoassay for VP16-213 in Plasma, Cancer Chemother. Pharmacol. 7:117-121.
44. Yamashita, K., Watanabe, K., Takayama, H., Ishibashi, M., and Miyazaki, H. 1987. Measurement of Plasma Etoposide by Radioimmunoassay, J. Pharm. Biomed. Anal. 5:11-20.
45. Hacker, M.P., Keve, S., and Ershler, W.B. 1984. Determination of VP16-213 Concentrations Using an Enzyme Linked Immunosorbent Assay (ELISA), Proc. Am. Assoc. Can. Res. 25:364.
46. Henneberry, H.P., Aherne, G.W., and Marks, V. 1988. An ELISA for the Measurement of VP16 (Etoposide) in Unextracted Plasma, J.

Immunol. Methods. 107:205-209.
47. Saita, T., Fujiwara, K., Kitagawa, T., Mori, M., and Takata, K. 1990. A Highly Sensitive Enzyme-linked Immunosorbent Assay for Etoposide Using β-d-Galactosidase as a Label, Cancer Chemother. Pharmacol. 27:115-120.
48. Van Opstal, M.A.J., Krabbenborg, P., Holthuis, J.J.M., Van Bennekom, W.P., and Bult, A. 1988. Comparison of Flow-injection Analysis with High-performance Liquid Chromatography for the Determination of Etoposide in Plasma, J. Chromatogr. 432:395-400.
49. Jungclas, H., Schmidt, L., Kohl, P., and Fritsch, H.W. 1993. Quantitative Matrix Assisted Plasma Desorption Mass Spectrometry, Int. J. Mass Spectrom. Ion Processes 126:157-161.
50. Littlewood, T.J., Hutchings, A.L., Bentley, D.P., and Spragg, B.P. 1984. High Performance Liquid Chromatographic Determination of Etoposide in Plasma Using Electrochemical Detection, J. Chromatogr. 336:434-437.
51. Eisenberg, E.J. and Eickholf, W.M. 1993. Determination of Etoposide in Blood by Liquid Chromatography with Electrochemical Detection. J. Chromatogr. B, -Biomed. Appl. 621:110-114.
52. Van Opstal, M.A.J., Van Der Horst, F.A.L., Holthuis, J.J.M., Van Bennekom, W.P., and Bult, A. 1989. Automated Reversed-phase Chromatographic Analysis of Etoposide and Teniposide in Plasma by Using On-line Surfactant-mediated Sample Cleanup and Column-switching, J. Chromatogr. 495:139-151.
53. Nielen, M.W.F., Sol, E., Frei, R.W., and Brinkman, U.A.T. 1985. Miniaturized Precolumn for On-line Trace Enrichment and Direct Plasma Injection in Narrow-bore Liquid Chromatography, J. Liq. Chromatogr. 8:1053-1070.
54. Gimmel, S. and Maurer, H.R. 1994. Concentration- and Time-dependent Cytostatic Effects of Methotrexate and Etoposide on L1210 Leukemic Cells in Vitro Demonstrated by a New Microperfusion Method, Anticancer Res. 14:129-135.
55. Allen, L.M. 1980. Analysis of 4' -Demethylepipodophyllotoxin-9-(4,6-O-ethylidene-β-D-glucopyranoside) by High-pressure Liquid Chromatography. J. Pharm. Sci. 69:1440-1441.
56. Farina, P., Marzillo, G., and D'Incalci, M. 1981. High-performance Liquid Chromatography Determination of 4'-Demethylepipodophyllotoxin-9-(4,6-O-ethylidene β-D-glucopyranoside) (VP 16-213) in Human Plasma. J. Chromatogr. 222:141-145.
57. Rideout, J.M., Ayres, D.C., Lim, C.K., and Peters, T.J. 1984. Determination of Etoposide (VP 16-213) and Teniposide (VM-26) in Serum by High-performance Liquid Chromatography with Electrochemical Detection, J. Pharm. Biomed. Anal. 2:125-128.
58. El-Yazigi, A. and Martin, C.R. 1987. Improved Assay for Etoposide in Plasma by Radial-compression Liquid Chromatography with Electrochemical Detection, Clin. Chem. (Winston-Salem, N. C.) 33:803-805.
59. Ploegmakers, H.H.J.L., Mertens, M.J.M., and Van Oort, W.J. 1985. Computerized Dynamic Voltammetric Detection in High-performance Liquid Chromatography. I. On-line Cyclic Voltammetry of the Antineoplastic Agents Etoposide and Teniposide, Anal. Chim. Acta 174:71-82.
60. Ploegmakers, H.H., Mertens, M.J., and Van Oort, W.J. 1987. Improved HPLC-ECD Analysis of Mitomycin C, Porfiromycin, VP 16-213 and VM 26 by Implantation of Software Filters, Anticancer Res. 7:1315-1319.
61. Shah, J.C., Chen, J.R., and Chow, D. 1989. Preformulation Study of Etoposide: Identification of Physicochemical Characteristics Responsible for the Low and Erratic Oral Bioavailability of Etoposide, Pharm. Res. 6:408-412.

62. Harvey, V.J. and Joel, S.P. 1985. High-performance Liquid Chromatography of Etoposide in Plasma and Urine, J. Chromatogr. 339:419-423.
63. Werkhoven-Goewin, C.E., Brinkman, U.A.T., and Frei, R.W. 1983. Automated Liquid Chromatographic Analysis of the Anti-tumorigenic Drugs Etoposide (VP 16-213) and Teniposide (VM 26), J. Chromatogr. 276:349-357.
64. Verweij, J., Lund, B., Beijnen, J., Planting, A., De Boer Dennert, M., Koier, I., Rosing, H., and Hansen, H. 1993. Phase I and Pharmacokinetics Study of Topotecan, a New Topoisomerase I Inhibitor, Ann Oncol. 4:673-678.
65. Lum, B.L., Kaubisch, S., Yahanda, A.M., Adler, K.M., Jew, L., Ehsan, M.N., Brophy, N.A., Halsey, J., Gosland, M.P., and Sikic, B.I. 1992. Alteration of Etoposide Pharmacokinetics and Pharmacodynamics by Cyclosporine in a Phase I Trial to Modulate Multidrug Resistance, J. Clin. Oncol. 10:1635-1642.
66. Floor, B.J., Klein, A.E., Muhammad, N., and Ross, D. 1985. Stability-indicating Liquid Chromatographic Determination of Etoposide and Benzyl Alcohol in Injectable Formulations, J. Pharm. Sci. 74:197-200.
67. Hersh, M.R. and Ludden, T.M. 1986. High-performance Liquid Chromatographic Assay for Etoposide in Human Plasma, J. Pharm. Sci. 75:815-817.
68. Miller, A.A., Stewart, C.F., and Tolley, E.A. 1990. Clinical Pharmacodynamics of Continuous-infusion Etoposide, Cancer Chemother. Pharmacol. 25:361-366.
69. Sessa, C., Zucchetti, M., Cerny, T., Pagani, O., Cavalli, F., De Fusco, M., De Jong, J., Gentili, D., McDaniel, C., Prins, C., Schacter, L., Winograd, B., and D' Incalci, M. 1995. Phase I Clinical and Pharmacokinetic Study of Oral Etoposide Phosphate, J. Clin. Oncol. 13:200-209.
70. Fleming, R.A. and Stewart, C.F. 1991. High-performance Liquid Chromatographic Determination of Etoposide in Plasma, J. Liq. Chromatogr. 14:1275-1283.
71. Schwinghammer, T.L., Fleming, R.A., Rosenfeld, C.S., Przepiorka, D., Shadduck, R.K., Bloom, E.J., and Stewart, C.F. 1993. Disposition of Total and Unbound Etoposide Following High-dose Therapy, Cancer Chemother. Pharmacol. 32:273-278.
72. Mans, D.R., Retel, J., Van Maanen, J.M.S., Lafleur, M.V., Van Schaik, M.A., Pinedo, H.M., and Lankelma, J. 1990. Role of the Semi-quinone Free Radical of the Anti-tumor Agent Etoposide (VP-16-213) in the Inactivation of Single- and Double-stranded Φ X174 DNA, Br. J. Cancer. 62:54-60.
73. Machida, M., Tanaka, S., and Nakamori, K. 1993. Simultaneous Determination of NK611, a Novel Water-soluble Derivative of Etoposide, and Its Metabolite (DeNK611) in Dog Plasma by Column-switching High-performance Liquid Chromatography, Biomed. Chromatogr. 7:82-85.
74. Allen, L.M., Tejade, F., Okonmah, A.D., and Nordqvist, S. 1982. Combination Chemotherapy of the Epipodophyllotoxin Derivatives, Teniposide and Etoposide, a Pharmacodynamic Rationale? Cancer Chemother. Pharmacol. 7:151-156.
75. Boos, J., Real, E., Schulze Westh of, Wolff, J., Euting, T., and Jurgens, H. 1992. Investigation of the Variability of Etoposide Pharmacokinetics in Children, Int. J. Clin. Pharmacol. Ther. Toxicol. 30:495-497.
76. Stiff, D.D., Schwinghammer, T.L., and Corey, S.E. 1992. High-performance Liquid Chromatographic Analysis of Etoposide in Plasma Using Fluorescence Detection, J. Liq. Chromatogr. 15:863-873.
77. Arbuck, S.G., Douglass, H.O., Crom, W.R., Goodwin, P., Silk, Y., Cooper, C., and Evans, W.E. 1986. Etoposide Pharmacokinetics in Patients with Normal and Abnormal Organ Function, J. Clin. Oncol. 4:1690-1695.
78. Mase, H., Hiraoka, M., Suzuki, A., and Nakanomyo, H. 1994. [Determination of New Anticancer Drug, Paclitaxel, in Biological

Fluids by High Performance Liquid Chromatography], Yakugaku Zasshi. 114:351-355.

79. Van Opstal, M.A.J., Blauw, J.S., Holthuis, J.J.M., Van Bennekom, W.P., and Bult, A. 1987. On-line Electrochemical Derivatization Combined with Diode-array Detection in Flow-injection Analysis, Rapid Determination of Etoposide and Teniposide in Blood Plasma, Anal. Chim. Acta 202:35-47.
80. Pfluger, K.H., Schmidt, L., Merkel, M., Jungclas, H., and Havemann, K. 1987. Drug Monitoring of Etoposide (VP16-213). Correlation of Pharmacokinetic Parameters to Clinical and Biochemical Data from Patients Receiving Etoposide, Cancer Chemother. Pharmacol. 20:59-66.
81. Kohl, P., Koppler, H., Schmidt, L., Fritsch, H.-W., Holz, J., Pfluger, K.H., and Jungclas, H. 1992. Pharmacokinetics of High-dose Etoposide after Short-term Infusion, Cancer Chemother. Pharmacol. 29:316-320.
82. Schacter, L.P., Igwemezie, L.N., Seyedsadr, M., Morgenthien, E., Randolph, J., and Albert, E.a., P. 1994. Clinical and Pharmacokinetic Overview of Parental Etoposide Phosphate, Cancer Chemother. Pharmacol. 34:58-63.
83. Zucchetti, M., De Fusco, M., Sessa, C., Frohlich, A., Reichert, S., and D' Incalci, M. 1994. High-performance Liquid Chromatographic Assay for the Determination of the Novel Podophyllotoxin Derivative Dime-thylaminoetoposide (NK611) in Human Plasma, J. Chromatogr. B, -Biomed. Appl. 654:97-102.
84. Liu, S.Y., Soikes, R., Chen. J., Lee, T., Taylor, G., Hwang, K.M., Lee, K.H., and Cheng, Y.C., GL331: a Novel Compound which Can Overcome Multiple Drug Resistance, Presented on May 24, 1993 as Abstract 2247 at the 84th AACR Annual Conference Held at Orlanda, Florida USA, May 19-22, 1993.

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Etoposide 及其相關化合物生物分析之研究現狀

李國雄 王惠康

美國北卡大學藥學院天然產物實驗室

摘　要

Etoposide (VP16) 於1983年經美國食品藥品管理局(FDA)批准上市，為治療睾丸腫瘤，Hodgkins 氏及非 Hodgkins 氏淋巴癌，小細胞肺癌，及其他各種惡性腫瘤之最有效藥物之一。VP16及其代謝產物的生物分析方法之開發，對其臨床藥物動力學，代謝降解和作用機理之研究都具有重要意義。本文討論了VP16及其代謝產物的生物分析方法之現狀及VP16的代謝途徑和作用機理研究之最新進展。目前，採用紫外，螢光或電化學檢出法的高效液相層析(HPLC)已廣泛應用於VP16及其代謝產物的生物分析，為臨床藥物動力學提供了靈敏及可靠的檢測手段。除此之外，利用現代免疫技術而開發的放射免疫測定法及酵素標記免疫吸附測定法也已相繼成功地應用於VP16及其代謝產物的生物分析。文末並討論了筆者實驗室新近開發成功的 epipodophyllotoxin 之新衍生物GL331的藥理及其臨床藥物動力學之研究進展。