azo bond reduction observed with orange G compared with brilliant crystal scarlet may be due to steric hindrance in the latter. Amaranth and azosulfamide had the highest rates of azoreductase activity due to the presence of more electron-withdrawing groups (sulfonates) in the ring system. Sulfachrysoidine, a lipid-soluble azo dye, as indicated by its large partition coefficient has a greater than anticipated rate of azo reduction due to the presence of the sulfonamido group. The potential for the formation of an intramolecular hydrogen bond between the hydroxyl or amino group at the *ortho* position of the aromatic rings and the azo nitrogen is common to all the dyes investigated in this study and, therefore, should not be a major contributing factor in the observed differences in the rates of azo bond reduction.

Because of the wide variety of structures of azo dyes, it is difficult to determine which physicochemical factor influences the rate of azo reduction by the hepatic azoreductase enzymes the most. This investigation used the 10,000×*g* supernatant fraction from liver homogenates as the enzyme source to determine if an overall generalization could be determined for the rates of reduction of azo dyes. The results of this investigation show that both the partition coefficient and the presence of electron-withdrawing substituents will influence the rate of reduction of the azo bond. Apparently, the larger the number of electron-withdrawing groups, the more rapid the rate of reduction by the hepatic azoreductase system.

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# High-Performance Liquid Chromatographic Analysis of the Semisynthetic Epipodophyllotoxins Teniposide and Etoposide Using Electrochemical Detection


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Received June 28, 1982, from the *Pharmacokinetics and Pharmacodynamics Section, St. Jude Children's Research Hospital, Memphis, TN 38101.* Accepted for publication December 13, 1982.



**Abstract** □ A high-performance liquid chromatographic (HPLC) assay was developed for the quantitation of two structurally similar and highly active anticancer drugs, etoposide (I) and teniposide (II), and their potential metabolites (hydroxy acid, picrolactone, and aglycone). The assay utilizes electrochemical detection, which imparts specificity and sensitivity sufficient to detect ≥20 ng/mL in plasma, urine, and CSF. The mean assay coefficients of variation were 5.1 and 8.1% for teniposide (10 μg/mL) and etoposide (5 μg/mL), respectively. The extraction efficiencies were 86% for etoposide, 70% for its hydroxy acid metabolite, 66% for teniposide, and 54% for the hydroxy acid of teniposide. The correlation coefficient of the multilevel standard curve was ≥0.995 over the concentration range of 0.05–50 μg/mL for the parent drugs and metabolites extracted from plasma. This method has been used to determine the concentrations of the parent drugs and their metabolites in the plasma, urine, and CSF of patients with cancer.



**Keyphrases** □ Etoposide—analysis with metabolites, high-performance liquid chromatography with electrochemical detection, human urine, plasma, and CSF □ Teniposide—analysis with metabolites, high-performance liquid chromatography with electrochemical detection, human urine, plasma, and CSF


Two relatively new and highly active antineoplastic drugs, etoposide {4′-demethylepipodophyllotoxin 9-[4,6-*O*-(*R*)-ethylidene-β-D-glucopyranoside], (I)} and teniposide {4′-demethylepipodophyllotoxin 9-[4,6-*O*-(*R*)-2-thenylidene-β-D-glucopyranoside], (II)} have clinical activity in childhood leukemias, lymphomas, neuroblastomas, brain tumors, and germ cell tumors and adult lung, brain, bladder, and testicular cancers as well as adult leukemias and lymphomas (1–5). The proposed metabolic scheme was derived from the known molecular transfor-




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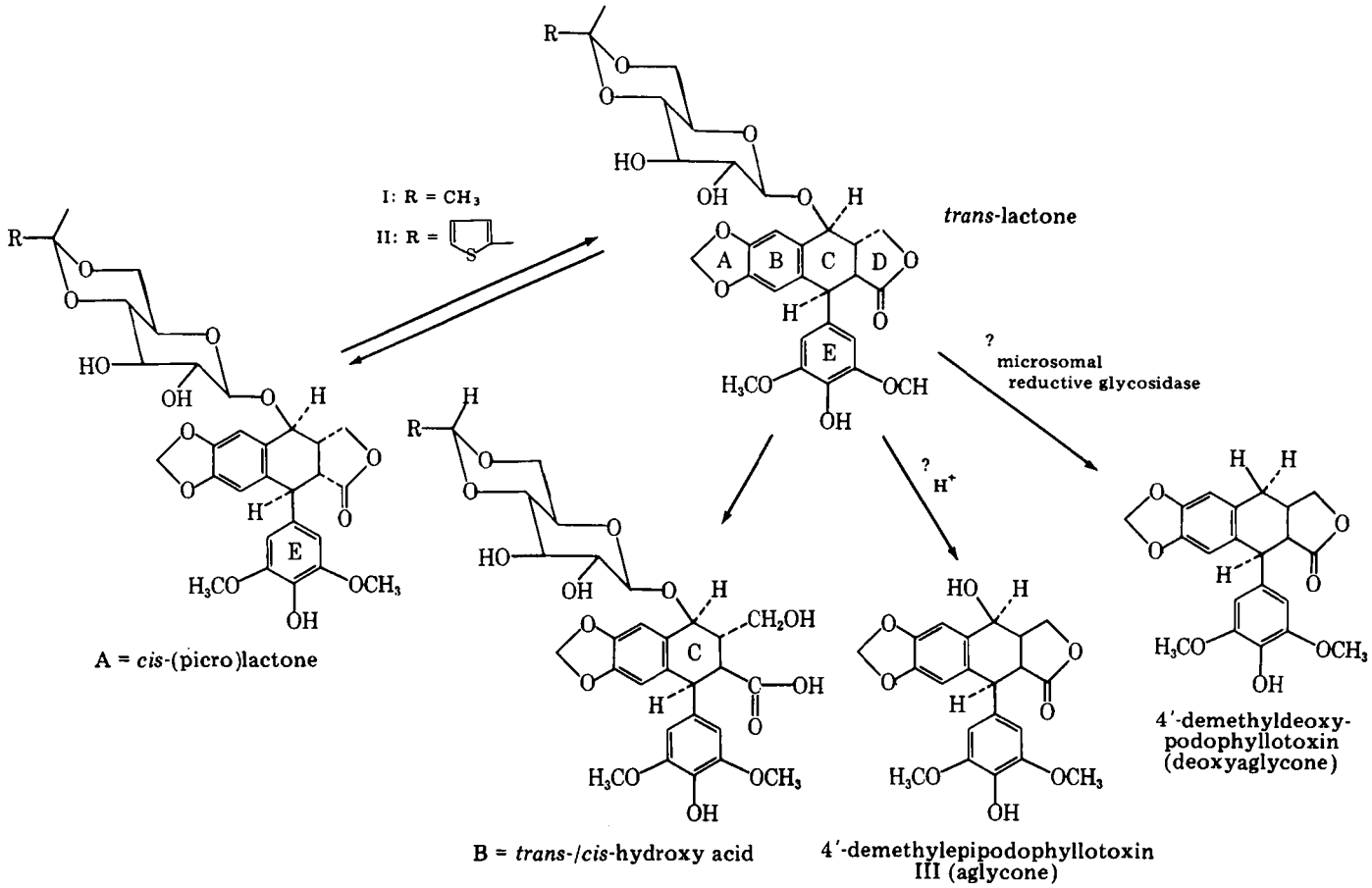


**Scheme I**—*Proposed metabolic transformations of the epipodophyllotoxin derivatives etoposide and teniposide.*

mations of podophyllotoxin, the compound from which these two drugs are synthesized (Scheme I). The biologically active molecules exist in a highly strained 2:3 *trans*-lactone ring that undergoes isomerization to a less active, more flexible, and less strained 2:3 *cis*-lactone or picro conformation hereafter referred to as picrolactones (IA, IIA) (6, 7). There are reports suggesting this isomerization takes place *in vivo* (8, 9). Initial Phase I pharmacokinetic studies using radiolabeled drug (10) measured total radioactivity in the chloroform-extractable layer and could not differentiate metabolites in human urine (11) and serum (12) that were later identified as chloroform-insoluble hydroxy acids (IB, IIB). Potential aglycones (III) formed by reductive or oxidative hydrolysis of the sugar moiety and glucuronide and/or sulfate conjugates are included as potential metabolites, although they have not yet been detected in biological fluids.

A new isocratic reverse-phase high-performance liquid chromatographic (HPLC) method utilizing the sensitivity and specificity of electrochemical detection was developed to quantitate these two drugs and their potential metabolites in the biological fluids of patients with cancer.

## EXPERIMENTAL

**Drugs, Reagents, and Equipment**—Etoposide (NSC 141540) and teniposide (NCS 122819) were gifts from the manufacturer[1]. The potential metabolites of etoposide and teniposide were synthesized as described elsewhere (12) or provided by other sources[2]. Drug standards were dissolved in methanol (1 mg/mL) and stored at −70°C. The standards were routinely checked for purity by TLC and HPLC. HPLC-grade methanol, acetonitrile, ethyl acetate, and chloroform were used throughout. Water was deionized, distilled, and filtered for HPLC use. Other chemicals and glassware were of standard laboratory quality. TLC plates included commercially available analytical silica gel (0.25 mm), preparative silica gel (2 mm), and reverse-phase $KC_{18}F$ (0.2 mm). The HPLC apparatus consisted of an isocratic pump[3], an injector[4], a 10-μm reverse-phase phenyl precolumn and analytical column[5], and a UV ab-

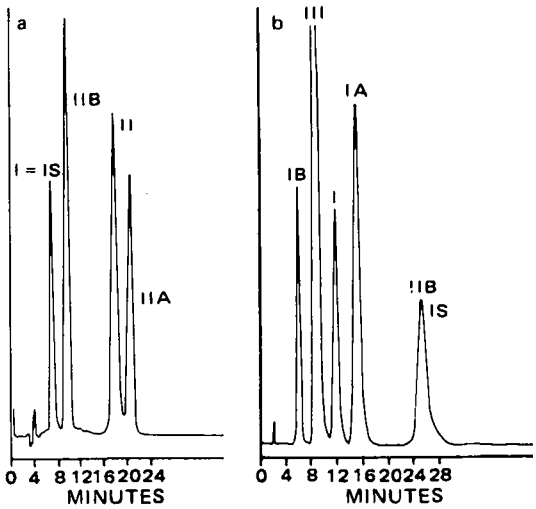


**Figure 1**—*Chromatographic separation of (a) teniposide (II) and its potential metabolites and (b) etoposide (I) and its potential metabolites. The HPLC conditions are described in the text.*

[1] Bristol Laboratories, Syracuse, N.Y.
[2] Dr. H. Stähelin, Sandoz LTD., Basel, Switzerland.
[3] Laboratory Data Control, Riviera Beach, Fla.
[4] Model 7125 with a 50-μL loop; Rheodyne, Berkeley, Calif.
[5] 4.6 mm × 250 mm, 10-μm, μBondapak phenyl; Water Associates, Milford, Mass.

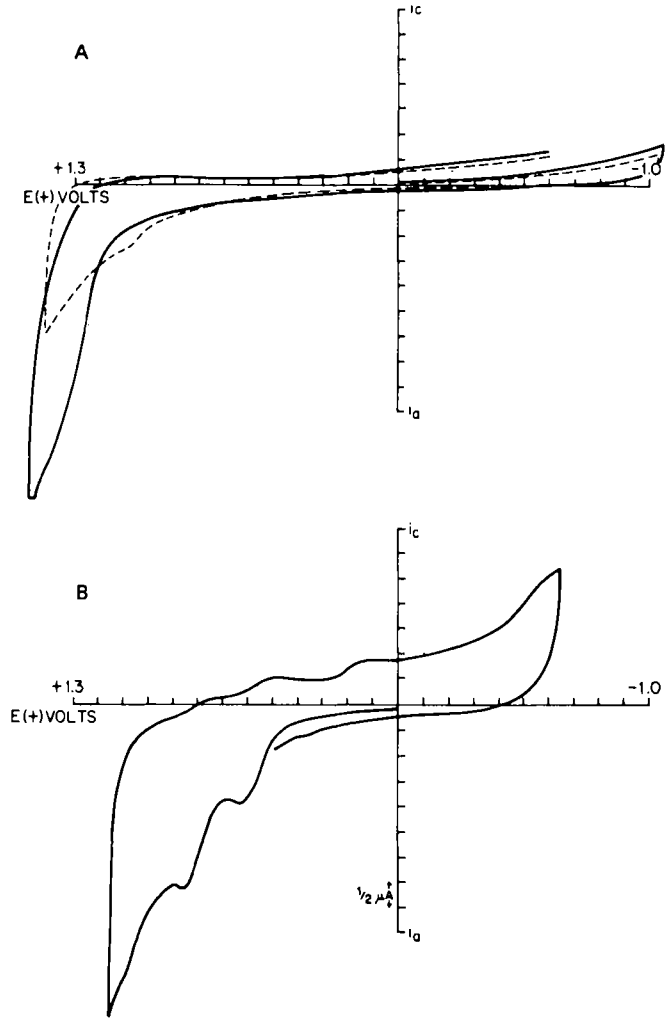


**Figure 2**—*Cyclic voltammograms of podophyllotoxin (A) and etoposide (B). Forward scan (anodic) from 0.0 applied potential; the broken line in A is the solvent scan.*

sorbance detector[6] connected in series to an amperometric (electrochemical) detector[7]. A dual-pen strip chart recorder was used for graphic display of the two detector outputs. Spectral identification of the metabolites was achieved by IR[8], $^1$H-NMR[9], and MS[10]. Cyclic voltammograms were performed in 70% sodium acetate (pH 5) and 30% 2-propanol. The scans initiated at 0.0 applied potential with the oxidative (anodic) preceding the reductive (cathodic) scan and returning to 0.0 potential[11].

**Liquid Chromatographic Conditions**—A prefiltered mobile phase of water–acetonitrile–acetic acid 68:30:2 at a flow rate of ~1 mL/min (1500 psi) through a 10-μm reverse-phase HPLC column[5] was used to resolve teniposide and its metabolites (Fig. 1A). Assay conditions for etoposide and its metabolites were similar, with a mobile phase consisting of water–acetonitrile–acetic acid (74:25:1) (Fig. 1B).

**Extraction of Biological Fluids**—*Teniposide*—To 0.5 mL of plasma, urine, or CSF was added 0.5 mL of saturated ammonium sulfate followed by 4 mL of ethyl acetate and 10 μL of a 100-μg/mL stock solution of etoposide (used as an internal standard). The sample was vortexed for 5 min and then centrifuged at 3000 rpm for 15 min. The upper (organic) layer was collected and set aside. The aqueous layer was reextracted with 4 mL of ethyl acetate by vigorously vortexing, then centrifuged at 3000 rpm for 15 min. The organic layer was combined with the previously collected ethyl acetate layer. This organic extract was dried under a gentle nitrogen stream, and the residue was reconstituted with 200 μL of methanol before HPLC analysis.

*Etoposide*—To 0.5 mL of plasma, urine, or CSF was added 5 μL of a 2.5-μg/mL stock solution of IIB (internal standard for I analysis) followed by saturated ammonium sulfate and double ethyl acetate extraction, as described above. The organic layer was decanted, dried under a nitrogen stream, and reconstituted with 200 μL of methanol just prior to injection.

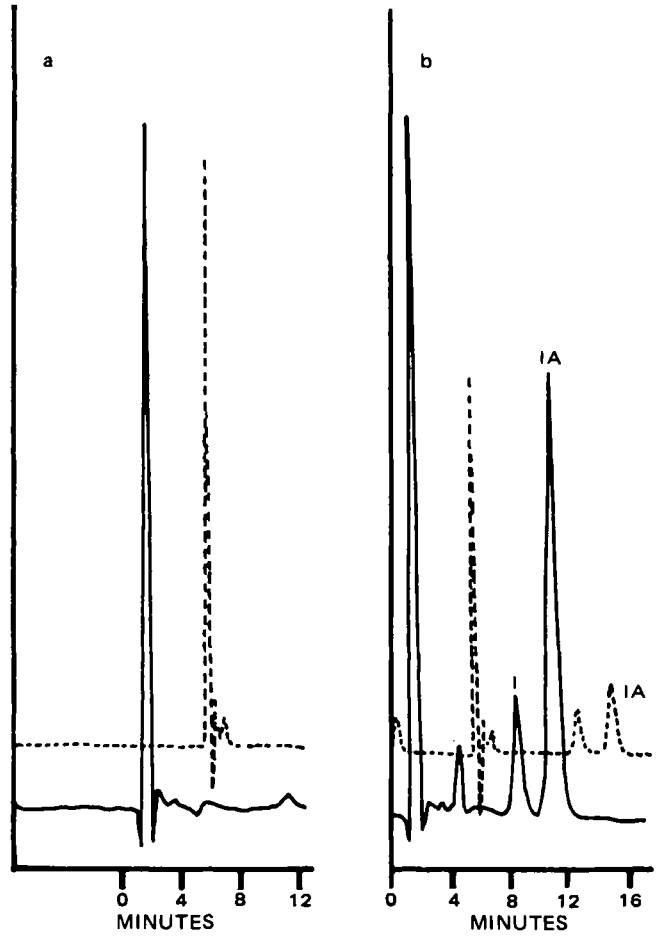


**Figure 3**—*Blank serum (a) and serum with etoposide and IA (b) showing relative UV (---) and electrochemical (—) response to the parent* trans*-lactone (I) and the picrolactone isomer (IA).*

**Extraction Efficiency**—To calculate the extraction efficiency, 10 aliquots of plasma containing either 10 μg/mL or 1 μg/mL of teniposide or etoposide and their respective metabolites were extracted, and the residues were reconstituted in 200 μL of methanol. Equivalent amounts (10 μg and 1 μg) of pure compounds in mobile phase were added to the residue of 1-mL blank plasma extracts. Extraction efficiency was calculated by the integrated area (or peak height) of the compound extracted, divided by the area (or height) of the same concentration of the compound added after extraction.

**Precision and Accuracy**—Teniposide or etoposide was added to 5 mL of pooled plasma (10 μg/mL) and thoroughly mixed. The sample was divided into 10 aliquots (0.5 mL each) and frozen at −70°C. All 10 samples were extracted and quantitated over a 3-d period. Daily ($n$ = 4) and day-to-day ($n$ = 3/d) coefficients of variation were calculated (expressed as percentage). Accuracy was determined by preparing samples containing unknown (blind) quantities of the compounds of interest. Percent recoveries were determined by two different operators on the same chromatographic system.

**Multilevel Calibration Curves**—Spiked samples in the concentration range of 0.05–50 μg/mL were extracted and each calibrator was analyzed in triplicate. Peak areas of the compounds were quantitated with the computing integrator, and peak heights were recorded by the strip-chart recorder attenuated to 100 mV. After chromatographic analysis, the peak areas, peak heights, peak area ratios (compound/internal standard), and peak height ratios were analyzed by linear regression against the known concentrations of the spiked samples.

[6] Model UV III, 284 nm; Laboratory Data Control, Riviera Beach, Fla.
[7] LC-4A Amperometric Detector; Bioanalytical Systems, Inc., West Lafayette, Ind.
[8] IR-20AX; Beckman, Berkeley, Calif.
[9] EM-930; Varian, Sunnyvale, Calif.
[10] Varian MAT; Varian, Sunnyvale, Calif.
[11] CV-1B Cyclic Voltameter; Bioanalytical Systems, Inc., West Lafayette, Ind.

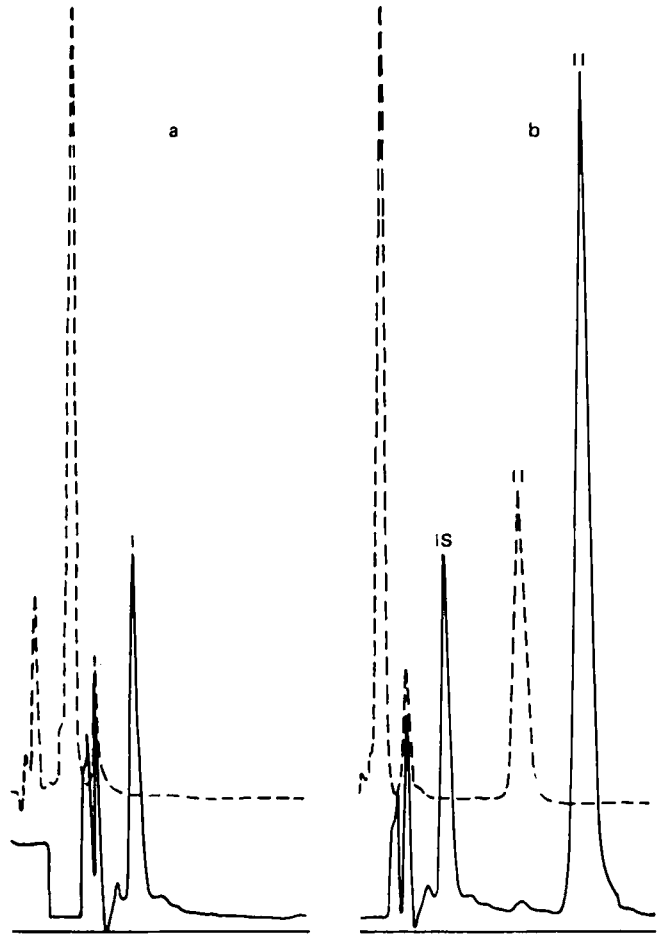


**Figure 4**—*Chromatograms of plasma obtained from a patient before (a) and 15 min after (b) a teniposide infusion. Key: (- - -) UV absorbance; (—) electrochemical response.*

**Application to Biological Fluids**—Blood (1–2 mL) was obtained in heparinized tubes prior to infusion and at 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h after the end of infusion and immediately placed on ice. Urine was collected for eight 6-h intervals in sterile containers and refrigerated.

## RESULTS

Cyclic voltammograms of etoposide and podophyllotoxin are reproduced in Fig. 2. Optimal potentials for electrochemical detection of teniposide and etoposide were 0.75 and 0.85 V, respectively. Podophyllotoxin was not oxidized and its cyclic voltammogram was not substantially different from the solvent scan. Since the 4′-demethylepidophyllotoxin (aglycone III) of teniposide and etoposide possesses electrochemical activity, the moiety responsible for the electroactivity of teniposide and etoposide may be the phenolic hydroxyl group on the E-ring of these molecules, which is not present on podophyllotoxin. However, the exact redox mechanism remains to be elucidated.

Typical HPLC profiles of teniposide, etoposide, and their potential metabolites are shown in Fig. 1A and B, respectively. The relative response of the parent drug and the picrolactone with UV and electrochemical detectors can be seen in Fig. 3. The UV monitor was at maximal detector sensitivity, and the electrochemical detector could have been adjusted 50-fold more sensitive. The enhanced electroactivity of the picrolactone over that of the *trans*-lactone is as yet unexplained. Table I shows the retention volumes ($V_R$) and capacity factors ($K'$) using the HPLC conditions noted above. Although separation of the *trans*-lactone from the picrolactone was not achieved using a nonpolar reverse-phase octadecylsilane ($C_{18}$ or ODS) bonded phase, separation was accomplished using a more polar bonded phase such as phenyl or cyanonitrile.

The extraction efficiency of teniposide and its potential metabolites were: IIB, 70%; II, 66%; and IIA, 55%. The extraction efficiency of etoposide and its potential metabolites were: IB, 90%; I, 88%; and IA, 80%. The retention volumes (or retention times) for components of either assay were reproducible with a day-to-day $CV$ of <2%. Quantitative precision ($CV$) for II (10 μg/mL, $n$ = 10) was 5.1%, while the $CV$ for I (5 μg/mL, $n$ = 10) was 8.1%. Day-to-day variations ($n$ = 3) for II, IIB, and IIA were 3.8, 7.6, and 4.2%.

**Table I—Retention Volumes ($V_R$) and Capacity Factors ($K'$) of I, II, and Their Potential Metabolites**

| Compound | $V_R$, mL | $K'$ |
|---|---|---|
| I[a] | 8 | 1.67 |
| II[a] | 26 | 7.67 |
| IIB[a] | 12 | 3.00 |
| IIA[a] | 30 | 9.00 |
| III[a] | 7.2 | 1.40 |
| III[b] | 14 | 3.67 |
| I[b] | 18 | 5.00 |
| IB[b] | 9 | 2.00 |
| IA[b] | 23 | 6.67 |
| IIB[b] | 30 | 9.00 |

[a] Retention data from a μBondapak phenyl column, 25 cm × 4.6 mm, with a mobile phase of water–acetonitrile–acetic acid (68:30:2) at a flow rate of 1 mL/min. [b] Retention data from a μBondapak phenyl column, 25 cm × 4.6 mm, with a mobile phase of water–acetonitrile–acetic acid (74:25:1) at 1 mL/min.

Least-squares regression analyses of teniposide and metabolite peak areas, peak area ratios, and peak height ratios to internal standard *versus* known concentration were linear from 0.05–50.0 μg/mL. The linear correlation coefficients of the best-fit line were >0.995 for the three methods of quantitation. Accuracy of the assay, determined by analysis of three prespiked unknown quantities of teniposide and metabolites was within ±5% of the true values.

A typical chromatogram of a preinfusion blank and 15-min postinfusion sample from one patient receiving teniposide (165 mg/m$^2$ over 40 min) is shown in Fig. 4. The concentration of teniposide in the sample chromatogram is 43.5 μg/mL. Figure 5 is a reproduction of a 15-min postinfusion chromatogram for a patient who received etoposide (250 mg/m$^2$ over 35 min); the plasma concentration shown was 72.2 μg/mL.

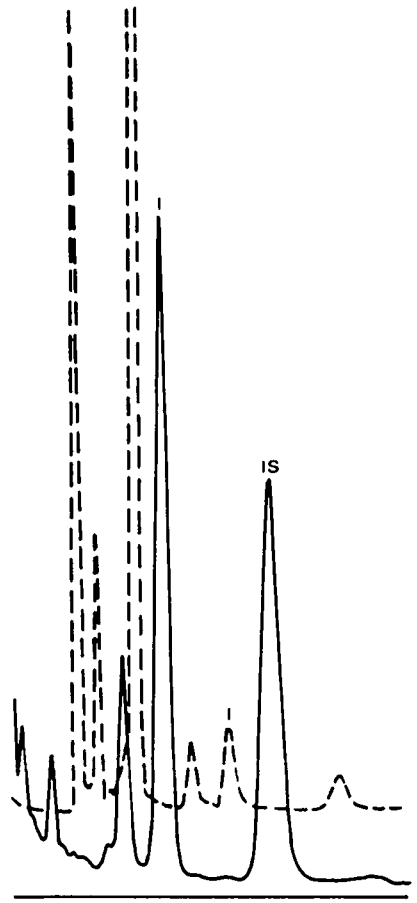


**Figure 5**—*Chromatogram of plasma obtained from a patient 15 min after an etoposide infusion. Key: (- - -) UV absorbance; (—) electrochemical response.*

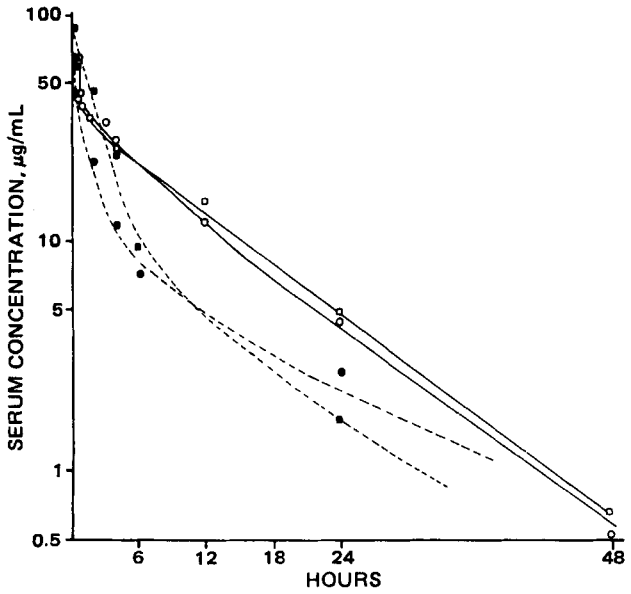


**Figure 6**—*Comparative plasma concentration* versus *time profiles of two patients who received etoposide at 250 mg/m² (■) and 200 mg/m² (●) and two patients who received teniposide at 165 mg/m² (□,○).*

Comparative plasma concentration *versus* time profiles of two patients are shown in Fig. 6.

## DISCUSSION

Both etoposide and teniposide are undergoing extensive clinical investigation for the treatment of various pediatric and adult malignancies. For this reason, a sensitive, specific, and simple analytical method for the determination of both etoposide, teniposide, and potential metabolic products of these drugs is necessary.

With a slight variation in the mobile phase, I, II, and all potential metabolites are well resolved from any coeluting endogenous substances. A relatively inexpensive electrochemical detector provides an almost 10-fold greater sensitivity over previously described HPLC–UV methods. The multilevel calibration curve (0.05–50.0 μg/mL) gave a linear correlation coefficient of $r \geq 0.995$, and the *CV* of each calibration point was ≤8%. The single step extraction of both parent drugs and metabolites is not optimal, and we are currently pursuing more efficient methods of extraction.

The hydroxy acids of teniposide and etoposide (IIB and IB) appear to be the metabolites detectable in plasma and urine, based on chromatographic peaks eluting in the region of the *cis*-hydroxy acids. The picrolactones of I and II (IA and IIA) were detected in the plasma and urine of some patients, but the concentrations never exceeded 5.0 μg/mL. The aglycone (III) has not been detected in any biological fluid to date.

Clinical investigations are currently underway utilizing this sensitive and specific HPLC assay for etoposide, teniposide, and their metabolites. These data should describe the pharmacokinetics of the drugs, further define components of systemic clearance (*i.e.*, metabolic and renal clearance), and evaluate any differences in the extent of metabolism of etoposide and teniposide in pediatric and adult patients.

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