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Simultaneous quantitation of etoposide and its catechol metabolite in human plasma using high-performance liquid chromatography with electrochemical detection

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Abstract

Etoposide, a highly active and widely used antineoplastic agent, is *O*-demethylated to its active catechol metabolite. A high-performance liquid chromatographic assay method for the simultaneous quantitation of etoposide and etoposide catechol in human plasma was established. Etoposide and etoposide catechol were extracted from plasma using chloroform and methanol followed by phase separation, evaporation of the organic phase, and reconstitution of the residue. Chromatography was accomplished using a reversed-phase phenyl analytical column (390 mm×3.9 mm I.D.) with a mobile phase of 76.6% 25 m*M* citric acid–50 m*M* sodium phosphate (pH 2.4)–23.4% acetonitrile pumped isocratically at 1 ml/min with electrochemical detection. The limit of detection for etoposide was 1.2 n*M* and for etoposide catechol was 0.2 n*M*. The precision (CV) for etoposide ranged from 0.7 to 3% and for the catechol metabolite from 1 to 6%; accuracy of predicted values ranged from 97 to 106% and 94 to 103%, respectively. The assay was linear from 0.1 to 10 μ*M* for etoposide and from 0.005 to 0.5 μ*M* for etoposide catechol in plasma. Recovery of etoposide and etoposide catechol ranged from 93 to 95% and 90 to 98%, respectively. Stability of etoposide and etoposide catechol in human plasma containing ascorbic acid stored at −70°C for one year was demonstrated.

Keywords: Etoposide; Etoposide catechol

1. Introduction

Etoposide, a semi-synthetic epipodophyllotoxin, is a highly active and widely used antineoplastic agent (Fig. 1) [1]. It has been shown to have activity in childhood leukemias, lymphomas, neuroblastomas, and brain and germ cell tumors, as well as in adult leukemias, lymphomas, lung, brain, bladder, and testicular cancers [2–4].

Metabolism of etoposide includes cytochrome P450-catalyzed *O*-demethylation in rodents [5–9] and in humans [10,11] of the dimethoxyphenol pendant ring, which forms the etoposide catechol (Fig. 1). Although this metabolite represents only a portion of the total drug that undergoes *O*-de-

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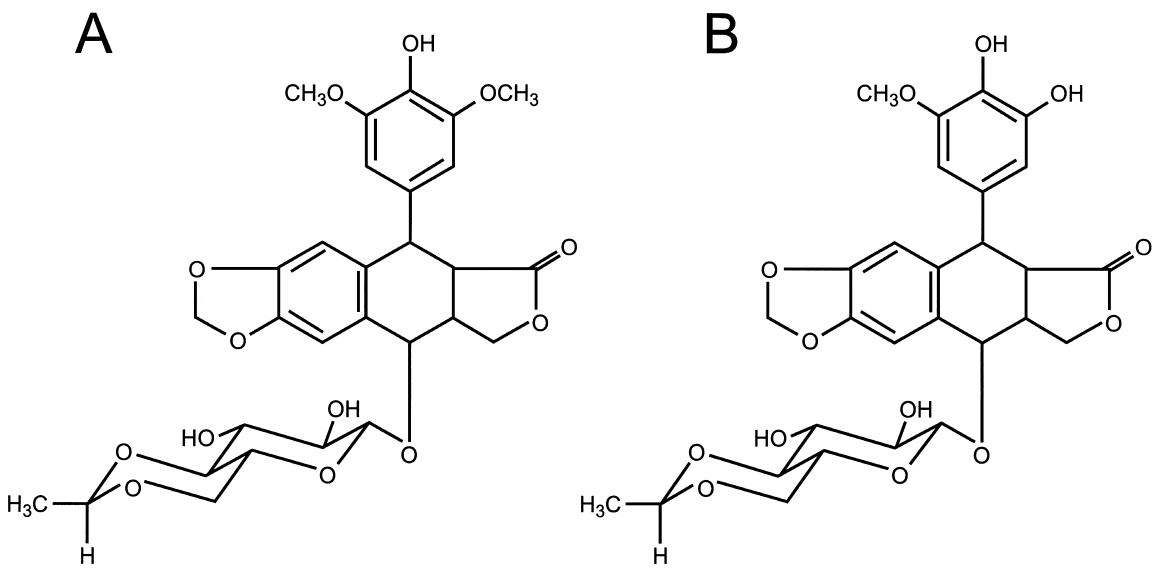

Fig. 1. Structure of (A) etoposide and (B) etoposide catechol.

methylation, as quinone and free-radical species are likely formed and interconversions among moieties may be rapid [12], measurement of the catechol is desirable for several reasons. First, the catechol is indicative of the overall *O*-demethylation pathway, and is detectable in urine [13] and plasma [14,15]. Also, these *O*-demethylated metabolites covalently bind to DNA and cellular protein, have intrinsic cytotoxic properties [5–9,16] as potent as the parent compound [8], and can enhance topoisomerase II mediated DNA cleavage [17,18]. Thus, inter-individual variability in the formation of reactive metabolites could have clinically important toxicological and possibly therapeutic ramifications.

Etoposide in human plasma has been quantitated by high-performance liquid chromatography (HPLC) with UV detection [19] and electrochemical detection [20–22] and etoposide catechol has been measured by HPLC with electrochemical detection [15]. We have developed an HPLC assay procedure using electrochemical detection for the simultaneous quantitation of etoposide and etoposide catechol that provides the desired selectivity with sufficient sensitivity to quantitate etoposide and its metabolite catechol simultaneously following administration of oral and intravenous etoposide to patients.

2. Experimental

2.1. Materials

Etoposide was initially a gift from Bristol Laboratories (Syracuse, NY, USA) and later purchased from Sigma (St. Louis, MO, USA). Etoposide catechol, which was synthesized by an oxidative *O*-demethylation of etoposide, followed by a reduction of the corresponding orthoquinones (vide infra), was generously provided by Dr. Josef Nemec (Chemical Synthesis and Analysis Laboratory, PRI/DynCorp, NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA). Reagent grade citric acid (monohydrate), ascorbic acid, and sodium phosphate, dibasic (anhydrous), and HPLC grade chloroform and acetone were obtained from Fisher Scientific (Somerville, NJ, USA), and HPLC grade acetonitrile and methanol were obtained from Burdick & Jackson (Muskegon, MI, USA). Distilled water was deionized and filtered through a Millipore Milli-Q system (Bedford, MA, USA). C_{18} Sep-Pak cartridges and 0.45 μm Nylon-66 filters used during mobile phase preparation were obtained from Waters (Milford, MA, USA) and Rainin (Woburn, MA, USA), respectively.

2.2. Instrumentation

The HPLC system consisted of a Shimadzu LC-10AT pump, SIL-9A autosampler, and CR501 integrator (Kyoto, Japan). Detection was performed with an Environmental Sciences Association Coulochem II electrochemical detector with a Model 5010 dual electrode analytical cell (electrode 1: +10 mV to screen interfering components, electrode 2: +370 mV to detect etoposide and etoposide catechol) (Bedford, MA, USA). A Model 5020 guard cell (+450 mV) was placed between the pump and the autoinjector to oxidize any electroactive components in the mobile phase. In order to measure the catechol concentration, which is relatively low compared to that of etoposide in biological samples, a time program was established to enhance detector sensitivity during collection of chromatographic data. The gain of the detector was set at 200 nA at the beginning of the data collection for etoposide catechol, and subsequently increased to 10 μA (after autozeroing the detector) at 18.5 min for etoposide. The acquisition delay of the detector was 10 s.

2.3. Chromatographic conditions

The stationary phase consisted of a Bondapak® Phenyl, 10 μm particle size, 390 mm×3.9 mm I.D., 125 Å pore size, analytical column (Waters). The prefiltered isocratic mobile phase consisted of 76.6% 25 m*M* citric acid–50 m*M* sodium phosphate (pH 2.4)–23.4% acetonitrile and was used at a flow rate of 1.0 ml/min. The water for the mobile phase was passed through a C_{18} Sep-Pak cartridge to reduce background components. The injection volume was 50 μl. The background current was less than 0.40 nA 3 h after the detector was turned on.

2.4. Preparation of stock and calibration solutions and control samples

Etoposide and etoposide catechol stock solutions (1 m*M* each) were prepared in methanol and stored at −70°C. Ascorbic acid was added to blank plasma to a final concentration of 10 m*M*. Etoposide and etoposide catechol methanolic stock solutions were used to prepare a standard plasma stock solution of 100 μ*M* etoposide–5 μ*M* etoposide catechol in plasma containing ascorbic acid. Plasma with ascorbic acid was spiked with various volumes of the 100 μ*M* etoposide–5 μ*M* etoposide catechol plasma stock solution to obtain six calibration solutions with concentrations ranging from 0.1 μ*M* etoposide–0.005 μ*M* etoposide catechol (58.9 ng/ml etoposide–2.86 ng/ml etoposide catechol) to 10 μ*M* etoposide–0.5 μ*M* etoposide catechol (5.89 μg/ml etoposide–0.286 μg/ml etoposide catechol). Controls were prepared in the same manner to make final concentrations of 1.0 μ*M* etoposide–0.05 μ*M* etoposide catechol (0.589 μg/ml etoposide–0.0286 μg/ml etoposide catechol) (low control) and 9.0 μ*M* etoposide–0.45 μ*M* etoposide catechol (5.30 μg/ml etoposide–0.257 μg/ml etoposide catechol) (high control).

2.5. Plasma extraction procedure

Seven milliliters of chloroform and 300 μl of methanol were added to 200 μl of plasma sample and rotated end-over-end for 5–10 min in acetone-rinsed glass tubes. The phases were separated by centrifugation at 200 *g* for 5 min and the upper aqueous layer was removed by freezing in a dry ice–acetone bath. The organic phase was cooled again in the dry ice–acetone bath to remove residual water and evaporated under a stream of nitrogen gas at room temperature. The residue was reconstituted in 200 μl of a solution of 30% 0.1 *M* acetate buffer (pH 5.0)–70% methanol (reconstitution buffer), vortex mixed for 10 s, and transferred to microcentrifuge tubes. The tubes were centrifuged at 7000 *g* for 2 min and the supernatant was transferred to autosampler vials just prior to HPLC analysis. The calibration, control, and patient samples were extracted in duplicate.

2.6. Stability

The stability of etoposide and etoposide catechol in plasma were evaluated following storage at −70°C. Whole blood was spiked with 5 μ*M* etoposide–0.1 μ*M* etoposide catechol and 25 μ*M*

etoposide–0.5 μ*M* etoposide catechol, respectively, then divided immediately into separate tubes containing no antioxidant, 20 m*M* ascorbic acid, 10 m*M* β-mercaptoethanol, and 1 m*M* DL-dithiothreitol (DTT). These antioxidant concentrations were the maximum levels which did not interfere with chromatographic data. The blood was spun at 1000 *g* for 5 min and plasma was collected. One set of plasma was assayed immediately. The other sets were stored at −70°C for two weeks, one month, five months, and one year prior to analysis.

2.7. Extraction efficiency

Aliquots of plasma containing 1.0 μ*M* etoposide–0.05 μ*M* etoposide catechol and 9.0 μ*M* etoposide–0.45 μ*M* etoposide catechol were extracted, and the residues were reconstituted in 200 μl of reconstitution buffer. Equivalent amounts of etoposide and etoposide catechol in methanol were evaporated under a stream of nitrogen gas, and the residues were reconstituted in the same volume of reconstitution buffer. Extraction efficiency was calculated by the dividend of the integrated peak heights of etoposide and etoposide catechol extracted, and those of the compounds which did not undergo extraction.

2.8. Precision and accuracy

Intra-day precision was evaluated by extraction and analysis of plasma samples containing two different concentrations of etoposide and etoposide catechol (ten samples containing 1.0 μ*M* etoposide–0.05 μ*M* etoposide catechol and ten samples of 9.0 μ*M* etoposide–0.45 μ*M* etoposide catechol) on one day. Inter-day precision was determined from evaluation of the two standard concentrations of etoposide and etoposide catechol in plasma in triplicate on five separate days. Accuracy was assessed by preparation of samples, by a separate technologist, which contained quantities of both compounds that were unknown to the assay operator.

2.9. Linearity and sensitivity

The linearity of the assay procedure was determined by calculation of a regression line using the method of least squares analysis. Limit of detection was defined as the peak height which was three-times larger than baseline noise.

2.10. Application to biological fluids

Blood (3 ml) was obtained in heparinized tubes containing 9±0.3 mg of ascorbic acid prior to etoposide infusion and at 1, 1.5, 2, 6, and 24 h after the end of infusion and immediately placed on ice.

3. Results

3.1. Detection optimization

The potentials of the first electrode (E_1) of the dual electrode cell was initially set at 0 mV, the second electrode (E_2) at +50 mV, and the guard cell (E_G) 75 mV higher than E_2. To optimize the potentials for electrochemical detection of both etoposide and etoposide catechol, the E_2 potential was increased in +30 mV increments and that of E_G increased correspondingly. Fig. 2 depicts the voltammogram of 9.0 μ*M* etoposide–0.45 μ*M* etoposide catechol in reconstitution buffer. The first channel of the integrator recorded the peak heights, which were proportional to electric current, of both compounds at the first electrode of the dual electrode cell for the same sample at different potentials. Optimal potentials of etoposide and etoposide catechol were +370 mV and +310 mV, respectively. Consequently, the potential of E_2 was set at +370 mV to provide maximal oxidation for etoposide and etoposide catechol and minimize oxidation of interfering compounds from plasma. The second channel of the integrator recorded the peak heights of both compounds at the second electrode. Oxidation of etoposide and etoposide catechol started at +50 mV and +10 mV, respectively. As a result, E_1 was set to +10 mV to minimize the loss of etoposide catechol before its detection.

3.2. Linearity and sensitivity

Linearity and sensitivity of the assay were evaluated based on four consecutive calibration curves constructed over a three month period. Least squares

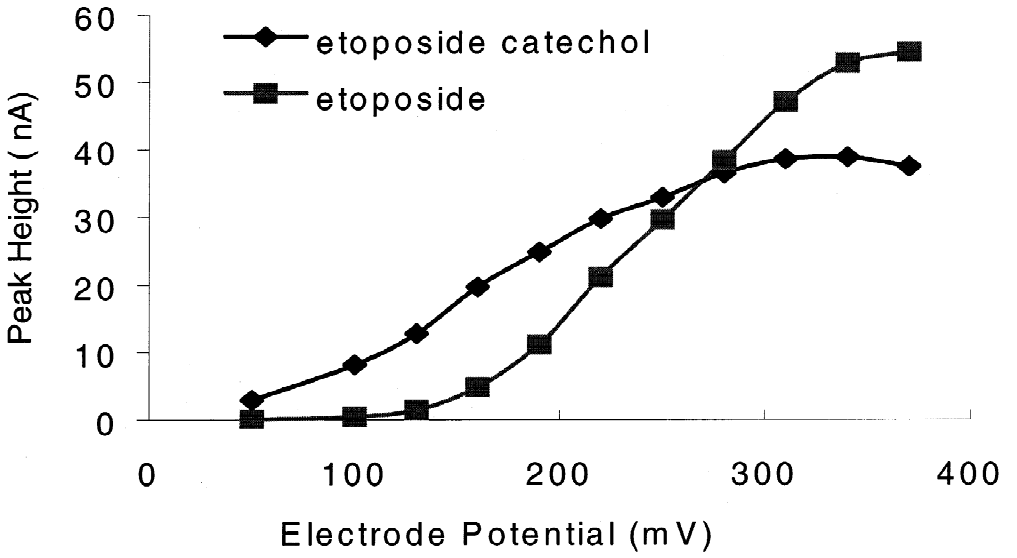

Fig. 2. Voltammogram of etoposide and etoposide catechol.

regression analyses of etoposide and etoposide catechol peak heights against known concentrations were linear from 0.1 μ*M* to 10 μ*M* (58.9 ng/ml to 5.89 μg/ml) and 0.005 μ*M* to 0.5 μ*M* (2.86 ng/ml to 0.286 μg/ml), respectively. Least squares regression data of the calibration curves are summarized in Table 1. The limit of detection for etoposide in human plasma was 1.2 n*M* (0.7 ng/ml) and for etoposide catechol 0.2 n*M* (0.1 ng/ml), corresponding to 0.06 and 0.01 pmol on column, respectively.

3.3. Recovery, precision, and accuracy

The recovery of etoposide and etoposide catechol from plasma is shown in Table 2. Recovery of over 90% was observed for both compounds at each evaluated concentration, based on six replicates. Precision data are summarized in Table 3. Intra-day precision was ≤0.7% for etoposide and ≤2.1% for etoposide catechol; inter-day precision was ≤3.3% for etoposide and ≤6.3% for etoposide catechol. Accuracy of the assay was within ±5% of the true values at 4.5 μ*M* and 8.5 μ*M* etoposide and 0.1 μ*M* and 0.35 μ*M* etoposide catechol, respectively. Because the low control concentration (1.0 μ*M* etoposide–0.05 μ*M* etoposide catechol) is 10-fold greater than the lowest standard concentration (0.1 μ*M* etoposide–0.005 μ*M* etoposide catechol), we also determined the precision and accuracy of the

Table 2
Recovery of etoposide and etoposide catechol from human plasma

Concentration (μ*M*)	Recovery (%)
Etoposide	
1.0	93.3±2.3
9.0	94.8±1.5
Etoposide catechol	
0.05	98.3±6.0
0.45	90.3±2.1

Table 1
Least squares regression analyses of standard curve data

	Slope (nA/μ*M*) (mean±SD)	*Y*-intercept (nA) (mean±SD)	Correlation coefficient (mean±SD)
Etoposide	2.724±0.123	0.184±0.052	0.9984±0.0010
Etoposide catechol	97.053±12.355	0.225±0.148	0.9983±0.0013

Table 3
Intra-day ($n=10$) and Inter-day ($n=3$/day for 5 days) precision and accuracy in plasma

Compound	Theoretical concentration (μM)	Mean observed concentration (μM)	Coefficient of variation (%)
Intra-day precision			
Etoposide	1.0	0.975	0.70
	9.0	9.56	0.69
Etoposide catechol	0.05	0.047	2.1
	0.45	0.46	1.1
Inter-day precision			
Etoposide	1.0	0.976	2.6
	9.0	9.11	3.3
Etoposide catechol	0.05	0.0487	4.4
	0.45	0.448	6.3

lowest standard concentration over a 3 month period ($n=8$). The mean etoposide concentration was 0.1032 μM (precision $\leq$2.4%) and the mean etoposide catechol concentration was 0.00524 μM (precision $\leq$20.5%).

3.4. Stability

The long-term stability of quality control samples was evaluated following storage at -70°C over a period of one year in the presence and absence of antioxidants (20 mM ascorbic acid, 10 mM β-mercaptoethanol, and 1 mM DTT) (Fig. 3). The peak height of etoposide was stable for the examined period of time, regardless of concentration and presence of antioxidant. In contrast, peak heights were lower at all time points for both low and high concentrations of etoposide catechol in the absence of ascorbic acid. Peak heights were comparable among samples without antioxidant and those with β-mercaptoethanol or DTT. Peak heights of etoposide catechol of samples without ascorbic acid decreased extremely rapidly (i.e., during sample preparation), as evident from the difference in peak heights at the first time point between samples with and without ascorbic acid. However, storage of samples at -70°C prevented further degradation over the one year period.

In addition, stability of reconstituted extracts of spiked plasma samples stored at room temperature in autosampler vials was assessed ($n=13$). Comparisons were made between replicates of samples injected with an average of 6.2 h in between injections. The mean (SD) percentage change over this time period for etoposide low control was -8.3% (10.9), etoposide catechol low control -2.8% (3.3), etoposide high control -2.5% (3.3), and etoposide catechol high control -2.3% (3.6).

3.5. Application of the assay

Typical chromatograms of a preinfusion blank and 6 h postinfusion sample from one patient receiving etoposide (300 mg/m^2 over 2 h) and a pre-dose blank and 1 h post-administration from another patient receiving oral etoposide (25 mg/m^2) are shown in Fig. 4. The plasma etoposide and etoposide catechol concentrations for the first patient were 6.53 μM and 0.149 μM, respectively, and for the second patient 0.765 μM and 0.0008 μM, respectively. A plasma concentration versus time profile of etoposide and etoposide catechol from a patient receiving intravenous etoposide (300 mg/m^2) is shown in Fig. 5.

4. Discussion

Etoposide is one of the most commonly used anticancer agents and is metabolized by *O*-demethylation to its active metabolite, etoposide catechol. Quantitation of both the parent drug and

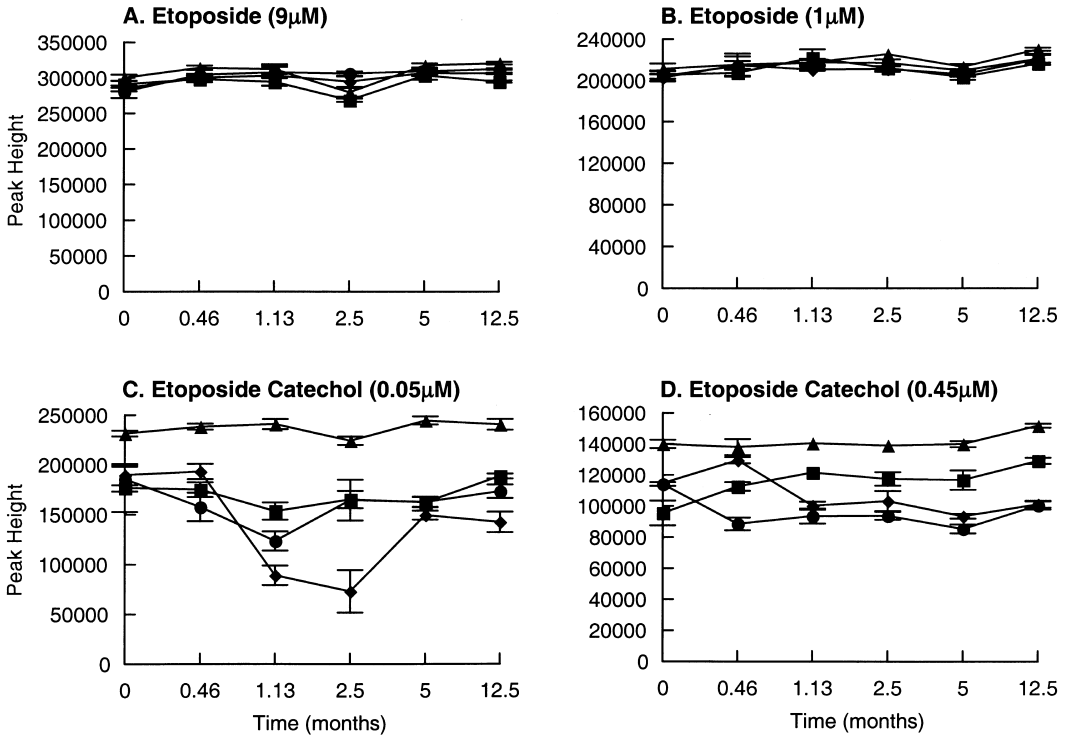

Fig. 3. Sample stability of etoposide and etoposide catechol in plasma at −70°C without addition of antioxidant (●) and with addition of 20 m*M* ascorbic acid (▲), 10 m*M* β-mercaptoethanol (■), or 1 m*M* DTT (♦) as described under the Experimental section.

catechol metabolite in plasma may provide important clinical information. However, obstacles in developing an assay to measure both compounds have included a feasible run time and ability to account for the large concentration differences between parent drug and metabolite. This is the first report of a simple, selective, and sensitive method developed to analyze both etoposide and etoposide catechol simultaneously in plasma of patients using reversed-phase HPLC with electrochemical detection.

The challenge of maintaining adequate accuracy and sensitivity for measuring etoposide catechol concurrent with the much higher concentrations of the parent etoposide has been managed by ensuring good extraction efficiency, clean background chromatograms, and time-programmable changes in both the detector and integratory settings. Etoposide and etoposide catechol were extracted from plasma with chloroform and methanol with an efficiency of over 90% for both compounds. The water used to prepare the mobile phase was passed through a C_{18} Sep-Pak Cartridge to reduce background current and the gain of the detector was increased (once chromatographic data for etoposide catechol was completed) for detection of etoposide. Such adjustments allowed not only the measurement of etoposide with a similar level of sensitivity, precision, and accuracy as previous HPLC methods using electrochemical [20–22], but also the simultaneous measurement of etoposide catechol to an even greater degree of sensitivity than that recently published by Stremetzne et al. [15] (our limit of detection of 0.2 n*M* versus published value of 17.4 n*M*). Moreover, it should be noted that our assay could be much more sensitive (10–50 fold) for etoposide if the gain were not set as high as we did (10 μA for etoposide versus 200 nA for etoposide catechol) for these routine clinical samples.

Peak heights were reproducible over several

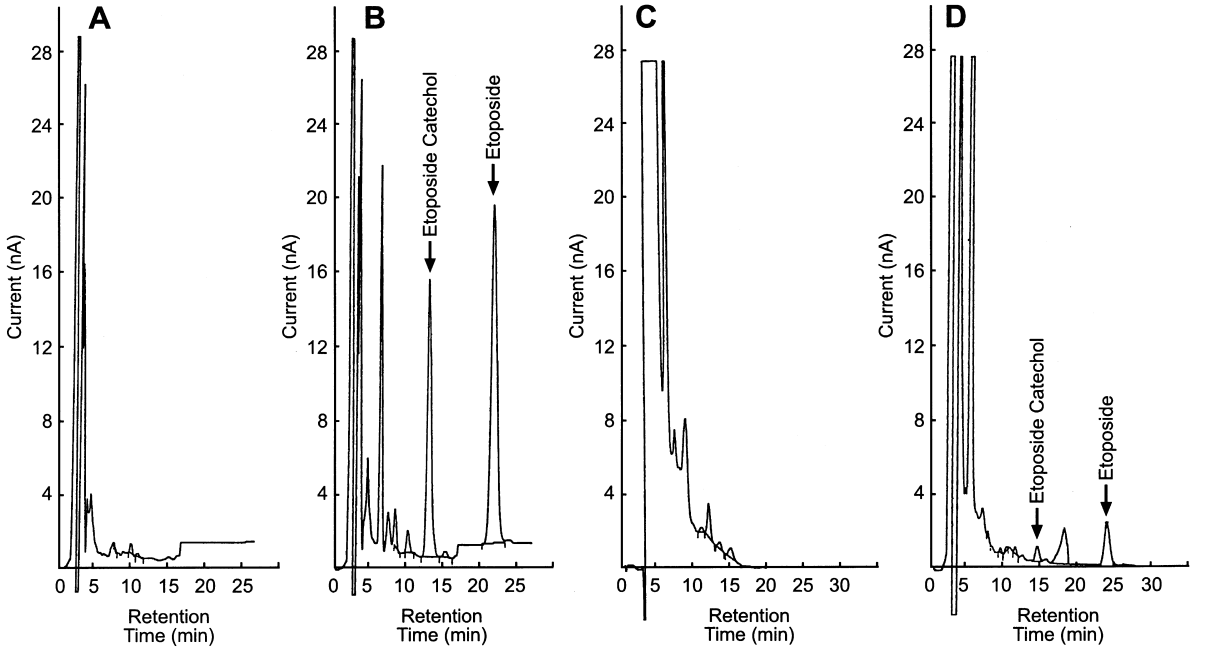

Fig. 4. Representative plasma chromatograms (A) before infusion and (B) 6 h after a 2 h infusion of 300 mg/m^2 etoposide to an acute lymphoblastic leukemia patient. The concentration of etoposide was 6.53 μ*M* and etoposide catechol was 0.149 μ*M*. Plasma chromatograms (C) before administration of etoposide and (D) 1 h after a 25 mg/m^2 oral dose. The concentration of etoposide was 0.765 μ*M* and etoposide catechol was 0.008 μ*M*. Integrator speed 2 mm/min, injection volume 50 μl, 1.0 ml/min flow rate. The sensitivity of the electrochemical detector was set 50 times higher for etoposide catechol than that for etoposide by the timeline program. No interfering peak was observed with the selected conditions.

months during assay development and sample analysis. The use of an internal standard was not necessary due to the excellent linearity of the calibration curves and intra-day and inter-day precision of control samples without internal standards. A calibration curve in human plasma gave a correlation coefficient of >0.998 for a concentration range of etoposide between 0.1 μ*M* to 10 μ*M* and etoposide catechol between 0.005 μ*M* to 0.5 μ*M*. Quality control samples were accurate to within 7% of theoretical concentrations.

Etoposide was stable in plasma regardless of the presence or absence of antioxidant. In contrast, there was a rapid decrease in peak height of etoposide catechol in samples with no antioxidant and samples in which β-mercaptoethanol or DTT were added. The only antioxidant we tested that protected the catechol from oxidation was ascorbic acid. Oxidation must occur during sample preparation, as the antioxidants were added to plasma prior to processing and the peak heights were diminished despite immediate assaying. We presume that such oxidation also takes place rapidly in vivo. Ex vivo, to protect the catechol from oxidation, ascorbic acid should be added before sample collection, and thus we have added ascorbic acid to the blood collection tubes to avoid instability of the catechol. Both etoposide and etoposide catechol plasma samples are stable for at least one year at −70°C.

Our assay has been applied to measure patient plasma concentrations of both etoposide and etoposide catechol following administration of etoposide. Our method permits the simultaneous determination of etoposide and etoposide catechol with a feasible run time and renders a means to manage the large concentration differences between

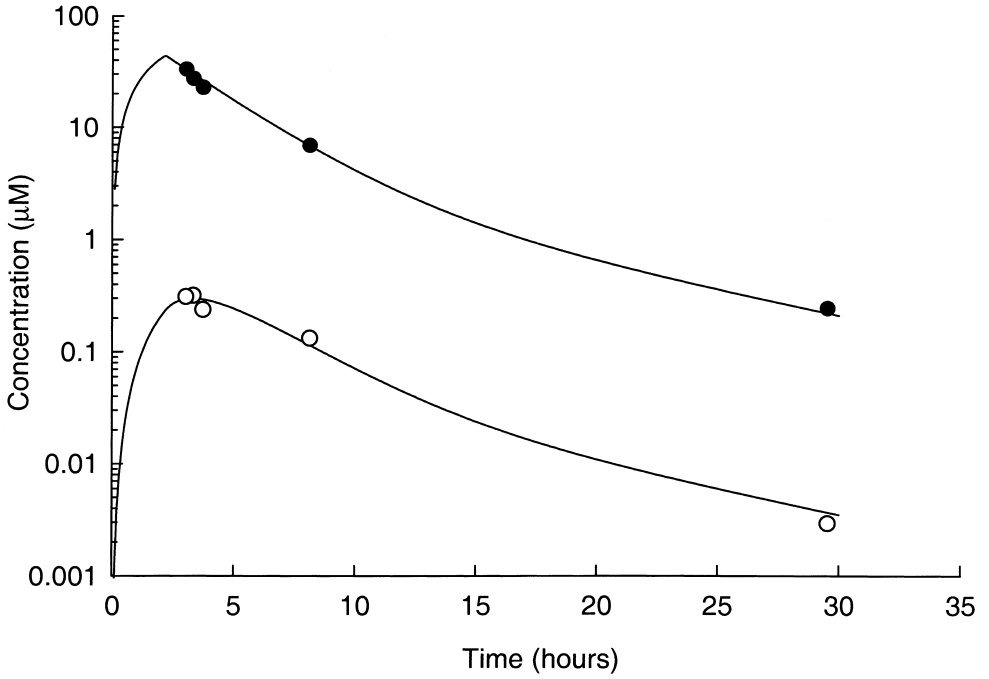

Fig. 5. Plasma concentration versus time profiles of etoposide (●) and etoposide catechol (○) after a 2 h intravenous infusion of etoposide 300 mg/m^2. The symbols represent measured plasma concentrations and the lines represent the best-fit curves using a Bayesian estimation algorithm for a first-order two-compartment structural pharmacokinetic model to simultaneously fit the parent drug and etoposide catechol data [13,14].

the parent drug and the catechol metabolite in clinical samples. This method has provided the simplicity, selectivity, and sensitivity necessary to describe full concentration–time profiles of etoposide and etoposide catechol of patients receiving etoposide.

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