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Simultaneous determination of etoposide and its catechol metabolite in the plasma of pediatric patients by liquid chromatography/tandem mass spectrometry

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The anticancer drug etoposide is associated with leukemias with *MLL* gene translocations and other translocations as a treatment complication. The genotype of cytochrome P450 3A4 (CYP3A4), which converts etoposide to its catechol metabolite, influences the risk. In order to perform pharmacokinetic studies aimed at further elucidation of the translocation mechanism, we have developed and validated a liquid chromatography/electrospray/tandem mass spectrometry assay for the simultaneous analysis of etoposide and its catechol metabolite in human plasma. The etoposide analog teniposide was used as the internal standard. Liquid chromatography was performed on a YMC ODS-AQ column. Simultaneous determination of etoposide and its catechol metabolite was achieved using a small volume of plasma, so that the method is suitable for pediatric patients. The limits of detection were 200 ng ml^{-1} etoposide and 10 ng ml^{-1} catechol metabolite in human plasma and 25 ng ml^{-1} etoposide and 2.5 ng ml^{-1} catechol metabolite in protein-free plasma, respectively. Acceptable precision and accuracy were obtained for concentrations in the calibration curve ranges 0.2–100 μg ml^{-1} etoposide and 10–5000 ng ml^{-1} catechol metabolite in human plasma. Acceptable precision and accuracy for protein-free human plasma in the range 25–15 000 ng ml^{-1} etoposide and 2.5–1500 ng ml^{-1} etoposide catechol were also achieved. This method was selective and sensitive enough for the simultaneous quantitation of etoposide and its catechol as a total and protein-free fraction in small plasma volumes from pediatric cancer patients receiving etoposide chemotherapy. A pharmacokinetic model has been developed for future studies in large populations.

KEYWORDS: etoposide; catechol metabolite; plasma; high-performance liquid chromatography; tandem mass spectrometry

INTRODUCTION

Etoposide (Fig. 1) is a semi-synthetic glycoside derivative of podophyllotoxin, originally an extract of the mandrake plant. It is used for the treatment of a variety of malignancies, such as small-cell lung cancer, testicular carcinoma, lymphoma, other solid tumors and several types of leukemia.[1] Teniposide, a structurally related epipodophyllotoxin analog of etoposide, has also been used as an anticancer drug (Fig. 1). One of the major pathways of etoposide metabolism involves cytochrome P450 3A4 (CYP3A4)-mediated formation of a catechol metabolite (Scheme 1).[2] Etoposide catechol can undergo sequential one-electron oxidations to form a semiquinone and a quinone (Scheme 1).[3,4] Subsequent redox cycling results in the generation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide,[4] which undergo Fenton chemistry to form hydroxyl radicals. The hydroxyl radicals either damage DNA directly[5] or induce the formation of lipid hydroperoxides, which break down to DNA-reactive aldehydic bifunctional electrophiles, malondialdehyde, 4-hydroxy-2-nonenal, and 4-oxo-2-nonenal (Scheme 1).[6] In addition, etoposide semiquinone and quinone can directly form adducts with DNA and protein molecules.[4,7–10] The primary mechanism of cytotoxicity of etoposide involves DNA topoisomerase II-mediated chromosomal breakage.[11,12] DNA topoisomerase II mediates essential changes in DNA topology by transient cleavage and religation of the double helix. Etoposide stabilizes the DNA cleavage and covalently linked enzyme intermediate by decreasing religation.[11,12] The resultant chromosomal breakage initiates apoptosis.[13]

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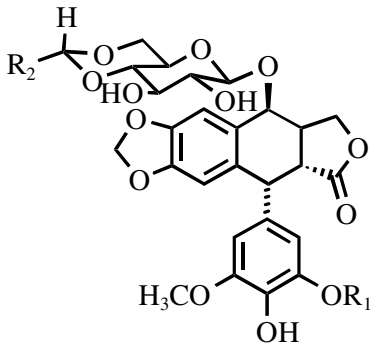
ROS and quinone formation, which results from etoposide metabolism has been suggested as an alternative mechanism for DNA damage.[4,7] The same mechanisms may be relevant to leukemia-associated chromosomal translocations.

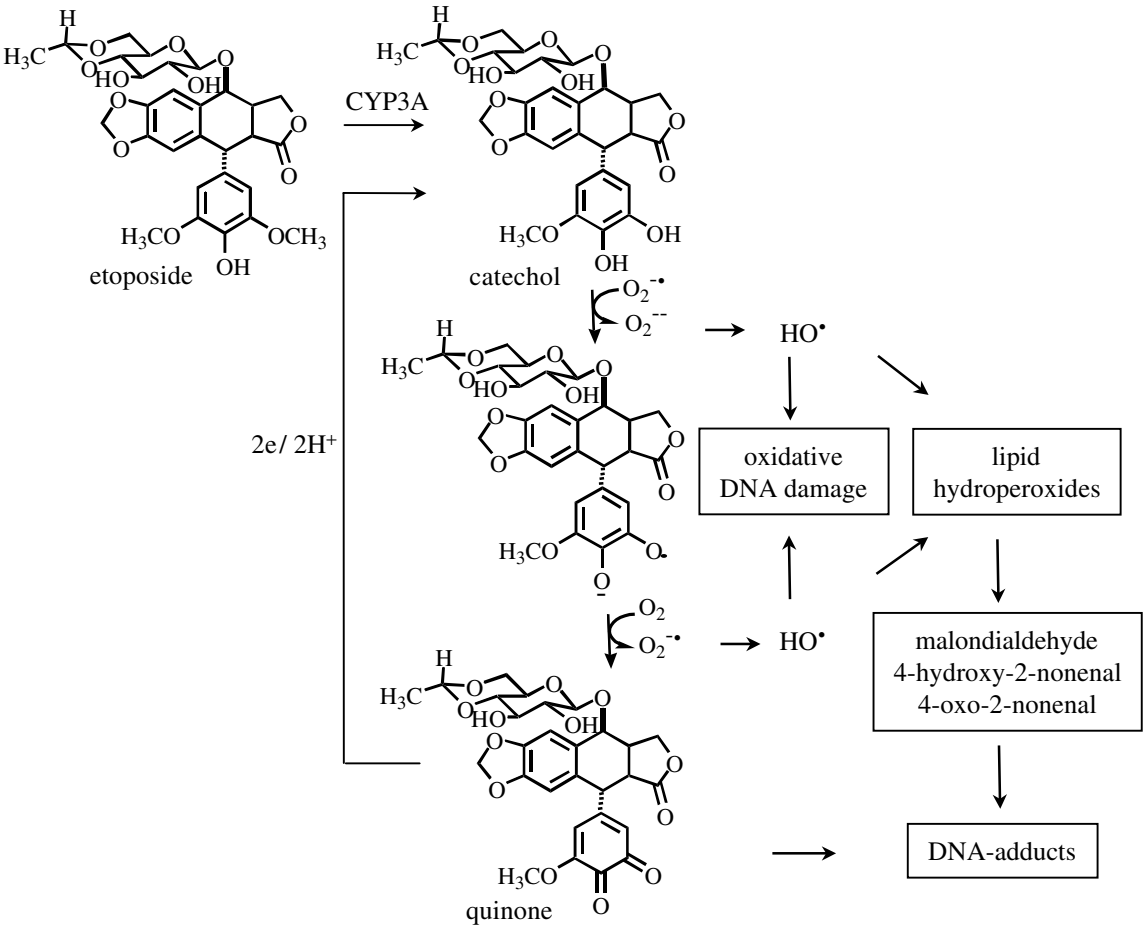
Etoposide is a substrate for metabolism by CYP3A4.[14] Therapy with etoposide is associated with leukemias with chromosomal translocations, especially translocations of the *MLL* gene at chromosome band 11q23, as a treatment complication.[15] The genotype of CYP3A4, which converts etoposide to its *O*-demethylated catechol metabolite, influences the risk.[15] Recently, we showed that etoposide catechol and etoposide quinone damage the *MLL* gene in a DNA-topoisomerase II-dependent, sequence-specific manner.[16] Furthermore, the N-7 position of guanine was critical to this damage, suggesting that it was a result of alkylation by the metabolites. It is not known whether individual differences in pharmacokinetics of etoposide or of its catechol metabolite, or both, are relevant to the leukemogenesis. Therefore, it is important to understand the contribution of the parent drug and its metabolites to the chromosomal breakage.

The objective of the present study was to develop a pharmacokinetic model that can ultimately be used to understand better the role of etoposide and its metabolites in the translocation process. Inter-patient differences in etoposide and etoposide catechol pharmacokinetics may provide insights into the CYP3A4 genotype–leukemia association. Pharmacokinetic monitoring may permit rational optimization of etoposide dose and schedule so that the risk of leukemia is reduced. Methods used previously for the determination of etoposide in human plasma include, high-performance liquid chromatography (HPLC) with UV detection,[17] fluorescence detection[18,19] and electrochemical detection.[20–23] HPLC methods based on electrochemical detection also have been used for the analysis of etoposide catechol in



Etoposide (MW=588)	R_1= CH_3	R_2= CH_3
Etoposide catechol (MW=574)	R_1= H	R_2= CH_3
Teniposide (MW=656)	R_1= CH_3	R_2= C_4H_3S

Figure 1. Structures of etoposide, etoposide catechol and teniposide (internal standard).



Scheme 1. Formation of etoposide catechol, etoposide semiquinone and etoposide quinone by metabolism of etoposide.

human plasma.[23,24] In patients with cancer, a substantial amount of plasma etoposide is non-covalently bound to plasma proteins with significant amount of inter-patient variation.[25] Protein binding, which varied from 80% to 97% (mean, 93%) for different individuals, appeared to be dependent upon serum albumin concentrations.[25] However, it is still unknown whether there are inter-individual differences in etoposide catechol binding. Furthermore, it is unclear whether there are changes in binding of etoposide and its catechol metabolite during multiple dosing regimens. The unbound (protein-free) fraction of etoposide correlates more closely with both toxicity and efficacy than the total drug (protein-free + non-covalently protein bound) concentration.[26] The concentrations of protein-free etoposide and protein-free etoposide catechol should be much lower than the total plasma concentrations. Therefore, highly sensitive analytical methodology with a wide dynamic range is necessary if both protein-free and total drug concentrations are monitored. We have developed a method with HPLC combined with electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) that simultaneously quantifies etoposide and its catechol metabolite as protein-free and total concentrations in human plasma samples. The method was successfully applied to pharmacokinetic studies in pediatric cancer patients receiving multiple-day intravenous infusions of etoposide.

EXPERIMENTAL

Chemicals and materials

Etoposide was purchased from Sigma (St. Louis, MO, USA). Teniposide was a gift from Bristol-Myers Squibb (Princeton, NJ, USA). Ascorbic acid, ammonium formate, dioxane, sodium metaperiodate and sodium borohydride were obtained from Aldrich (Milwaukee, WI, USA). HPLC-grade water, formic acid, sodium sulfate and dichloromethane were obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade acetonitrile was obtained from B&J (Muskegon, MI, USA). Blank human plasma and protein-free plasma were purchased from Biological Specialty (Landsdale, PA, USA). Diethyl ether was obtained from VWR (Bridgeport, NJ, USA), and ammonium sulfate from Mallinckrodt (Paris, KY, USA).

Synthesis of etoposide catechol (epipodophyllotoxin catechol glucoside)

Etoposide quinone was synthesized according to the patent of Nemec[27] with minor modifications. Etoposide (0.130 g, 0.221 mmol) was dissolved in dioxane (2.4 ml) and water (4.7 ml). One portion of 1.4 ml of 0.5 M sodium metaperiodate ($NaIO_4$) was added with stirring to this solution. The reaction mixture was kept in the dark at $10 \pm 4\,^{\circ}C$ for 110 min then saturated with solid $(NH_4)_2SO_4$. Etoposide quinone was extracted with dichloromethane (1 × 4 ml, then 5 × 2 ml). The organic phases were combined, washed with water (5 × 2 ml), and dried with Na_2SO_4. After evaporation to dryness under a stream of nitrogen, the residue was dissolved in a minimum volume of dichloromethane. Diethyl ether was added to the dichloromethane solution to initiate crystallization. After centrifugation at 3000 rpm for 10 min, the supernatant was discarded and the residue was washed with diethyl ether three times. Pure etoposide quinone was kept inside an aluminum foil-wrapped flask, dried under vacuum overnight and stored at room temperature in the dark. It was converted to etoposide catechol by the method of Relling *et al*.[28] Briefly, etoposide quinone (0.296 g, 0.505 mmol) was dissolved in dioxane (5 ml) and water (10 ml) under an atmosphere of nitrogen (sealed Aldrich AtmosBag). A solution of 1 M sodium borohydride ($NaBH_4$; 2 ml) was then added dropwise with stirring. The solution was kept on an ice–water bath and more 1 M $NaBH_4$ solution (0.8 ml) was added dropwise. After 10 min, the solution was treated with 1.2 ml of 1 M HCl to stop the reaction. The reaction mixture was extracted with dichloromethane (4 ml once, and then 4 × 2 ml), the organic phases were combined, washed with water (5 × 2 ml), and dried over Na_2SO_4. After evaporation to dryness under a stream of nitrogen, the residue was recrystallized with dichloromethane–diethyl ether as described for etoposide quinone. Recrystallized etoposide catechol was stored in the dark at room temperature.

Liquid chromatography

HPLC separation was performed on a Waters (Milford, MA, USA) model 2690 Alliance HPLC system with a YMC (Wilmington, NC, USA) ODS-AQ analytical column (150 × 2.0 mm i.d., 3 μm) preceded by a precolumn filter (2 μm; Alltech, Deerfield, IL, USA). The mobile phases consisted of 5 mM $HCOONH_4$ and 0.1% aqueous formic acid solution with 10% acetonitrile as A and 90% acetonitrile as B. The analytes were eluted with a gradient at a flow-rate of 0.2 ml min^{-1}. A set of 50 samples (200 μl of each) was run at one time with the autosampler maintained at 5 °C. When the samples were analyzed, the column effluent was diverted to waste for the first 2 min and the last 10 min in order to minimize contamination of the mass spectrometer.

Mass spectrometry

ESI-MS analysis was performed using a Finnigan TSQ7000 triple-quadrupole mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with a Finnigan electrospray ionization source. The mass spectrometer was operated in the positive ion mode and nitrogen was used as both sheath gas (70 psi) and auxiliary gas (25 units). The source was maintained at 200 °C and the needle potential at 4.5 kV. The multiplier voltage was set at 1500 V and argon was used as the collision gas at 3.5 mTorr (1 Torr = 133.3 pa). The mass spectrometer, configured in the selected reaction monitoring (SRM) scan mode, monitored the transitions m/z 592.2 → 229.2 for etoposide catechol and m/z 606.2 → 229.2 for etoposide from 0.0 to 7.0 min, and m/z 674.2 → 229.2 for teniposide (internal standard) from 7.0 to 10.0 min with a scan time of 0.5 s. The collision energy was optimized to −32 eV for the determination of both etoposide and teniposide and to −25 eV for the determination of etoposide catechol. The data system was controlled using Finnigan ICIS 8.3.0 software and Finnigan LCQuan V1.2 was used to calculate peak areas.

Catechol stability

In order to test the stability of the analytes in the injection solutions, etoposide, etoposide catechol and teniposide were spiked into 34% aqueous acetonitrile solutions containing 0, 0.2, 2 and 50 mM ascorbic acid. Samples were kept at 5 °C and taken at certain time points for analysis. In order to test the stability of etoposide catechol during the sample preparation, etoposide catechol was spiked into blank plasma and left at room temperature. Samples were taken at 0, 0.5, 1, 2, 3 and 4 h for analysis.

Sample preparation

Etoposide, etoposide catechol and teniposide were dissolved in methanol. Working standard solutions were prepared by diluting the stock standard solution (1 mg ml^{-1}) with methanol. Concentrations of the final plasma standard solutions were one hundredth of the working standard concentrations so that the final concentration of methanol was only 1% of the total volume in plasma samples. Standard plasma samples were prepared by spiking etoposide and etoposide catechol into blank human plasma. All the standard samples were aliquoted and stored at −80 °C until they were used. The concentration of the internal standard (teniposide) was 10 μg ml^{-1}. All plasma (regular or protein-free) standards and patient samples were thawed on ice before analysis. For the quantitation of total etoposide and its catechol in human plasma samples, plasma standard solution (50 μl), teniposide (internal standard, 20 μl, 10 μg ml^{-1}), freshly made ascorbic acid solution (20 μl, 50 mM) and acetonitrile (260 μl) were added to an Eppendorf centrifuge tube. After vortex mixing for 5 min, the sample tubes were centrifuged in an Eppendorf 5415C centrifuge at 14000 rpm for 5 min. The supernatant of each sample was transferred into another centrifuge tube and evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 200 μl of 34% acetonitrile in water (initial mobile phase for HPLC gradient). The sample solutions were filtered through a Costar spin-X filter (2 μm) and transferred to autosampler vials, and 20 μl of each solution were injected into the HPLC system.

For the determination of protein-free etoposide and its catechol metabolite, 500 μl human plasma samples were filtered through Millipore (Bedford, MA, USA) Centrifree filters and spun in a Beckman (Palo Alto, CA, USA) GS-6KR centrifuge at 3920 rpm in a fixed-angle rotor (∼1900 *g*) for 60 min at 10 °C to remove protein and protein-bound etoposide and etoposide metabolites. The clear, colorless filtrate was used for the quantitation of protein-free fractions of etoposide and etoposide catechol. The procedure for the protein-free fraction analysis was the same as for total plasma except that 200 μl of protein-free plasma were used instead of 50 μl of plasma and 800 μl of acetonitrile were used.

Calibration curves

The total and protein-free fractions of etoposide and its catechol metabolite in human plasma were analyzed separately. Calibration curves were prepared by adding known amounts of etoposide and etoposide catechol to drug-free human plasma at concentrations of 0.2–100 μg ml^{-1} etoposide and 10 ng ml^{-1}–5 μg ml^{-1} etoposide catechol for total (bound + free) analysis. For the determination of protein-free concentrations, known amounts of etoposide and etoposide catechol were added to drug- and protein-free human plasma at concentrations of 25 ng ml^{-1}–15 μg ml^{-1} for etoposide and at 2.5 ng ml^{-1}–1500 ng ml^{-1} for etoposide catechol. The data were analyzed by using LCQuan Ver. 1.2. Quantitation was achieved by plotting the peak area ratios of the analytes to the internal standard versus concentration followed by linear regression analysis with a weighting factor of $1/x^2$. A water blank, plasma blank and control sample (blank plasma spiked with internal standard only) were also prepared and analyzed with each calibration curve.

Accuracy and precision

The method for the analysis of both total and protein-free fractions of etoposide and its catechol in human plasma was validated by analyzing the quality control (QC) samples on three separate days. For total (bound + free) etoposide and etoposide catechol determination, the QC samples were prepared by adding known amount of etoposide and etoposide catechol to drug-free human plasma. The QC samples contained a ratio of etoposide to etoposide catechol of 20 : 1 with concentrations of 200 and 10 ng ml^{-1} (lower limit of quantitation, LLQ), 500 and 25 ng ml^{-1} (lower quality control, LQC), 5000 and 250 ng ml^{-1} (middle quality control, MQC) and 50 000 and 2500 ng ml^{-1} (high quality control, HQC). For protein-free etoposide and etoposide catechol, the QC samples were prepared by adding known amount of etoposide and etoposide catechol to drug- and protein-free human plasma. The QC samples contained a ratio of etoposide to etoposide catechol of 10 : 1 with concentrations of 25 and 2.5 ng ml^{-1} (LLQ), 200 and 10 ng ml^{-1} (LQC), 1000 and 100 ng ml^{-1} (MQC) and 10 000 and 1000 ng ml^{-1} (HQC). The QC samples were analyzed on three separate days and the LLQ samples were analyzed on one day. The accuracy of this method was determined by comparing the means of the measured concentrations with the theoretical concentrations in the quality control samples and presented as a percentage. The intra-day precision was expressed as the relative standard deviation (RSD) of the sample replicates over their mean values at each concentration within the same validation day. The inter-day precision was expressed as the RSD of three different validation days.

Cancer patient treatment and plasma sample collection

The studies were approved by the IRBs of the Children's Hospital of Philadelphia and the University of Pennsylvania. In a typical clinical study, a pediatric cancer patient received a 100 mg m^{-2} dose of etoposide as a 1 h infusion daily for 5 days. On days 1 and 5, blood samples were taken at selected points before and after completion of the infusion. The blood samples were kept on ice and centrifuged at 3000 rpm for 10 min at 10 °C. Plasma was aliquoted and stored in a freezer at −80 °C immediately. To obtain protein-free plasma, plasma (0.5 ml) was added to a Centrifree filter (Millipore) and centrifuged at 10 °C for 60 min at 1900 *g*. The protein-free plasma samples were also stored at −80 °C.

Pharmacokinetic modeling

All modeling was conducted using the NIH-based WinSAAM kinetic modeling software.[29] This uses a weighted least-squares approach to fit the etoposide and catechol data simultaneously, producing direct estimates of the adjustable rate constants. For all data fitting, rate parameters were considered resolved when their fractional standard deviations (FSDs) were <0.5 (or 50%). In fact, no estimated parameter had an FSD >20%.

RESULTS AND DISCUSSION

Mass spectrometry

Full-scan mass spectra of etoposide, etoposide catechol and teniposide showed abundant ions at m/z 606, 592, and 674, respectively. These ions corresponded to ammonium adduct ions, $[M + NH_4]^+$, for each of the compounds. Product ion spectra of $[M + NH_4]$ m/z 606 (etoposide), m/z 592 (etoposide catechol) and m/z 674 (teniposide) at a collision energy of −25 eV are shown in Fig. 2. Product ions were formed through two major pathways: loss of the sugar moiety and the phenolic side-chain. Proposed fragmentation pathways of all three compounds are shown in Fig. 3. In order to achieve the highest sensitivity for selected reaction monitoring (SRM) analysis, the signal intensities of the product ions from each compound were optimized by changing the collision energy. For etoposide, the transition of m/z 606 → 229 was maximized with a collision energy of −32 eV; for etoposide catechol, the transition of m/z 592 → 229 was maximized with a collision energy of −25 eV; and for teniposide, the transition of m/z 674 → 383 was maximized with a collision energy of −32 eV. The argon collision gas pressure was optimal at 3.5 m Torr for all three analytes.

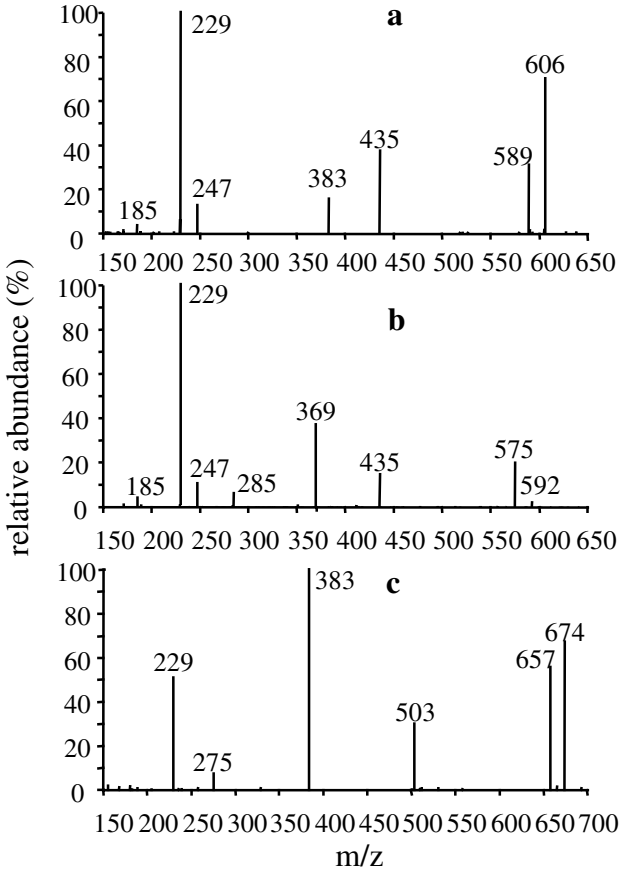


Figure 2. Product ion spectra of (a) etoposide, (b) etoposide catechol and (c) teniposide (internal standard) at a collision energy of −25 eV with argon at 3.5 mTorr. The parent ions of etoposide, etoposide catechol and teniposide were at *m/z* 606, 592 and 674, respectively, owing to the formation of ammonium adduct ions. A mobile phase of acetonitrile–aqueous solution containing 5 mM ammonium formate and 0.1% formic acid was used.

Liquid chromatography

Separation of the analytes and internal standard was accomplished in 10 min using a gradient of acetonitrile–water containing 5 mM ammonium formate and 0.1% formic acid. Representative SRM chromatograms are shown for blank human plasma spiked with the teniposide internal standard [Fig. 4(a)] and blank protein-free plasma spiked with internal standard [Fig. 5(a)]. There were no interfering peaks at the retention times for etoposide or etoposide catechol. No interfering peaks were observed at the retention time of the internal standard (data not shown). Representative chromatograms are also shown for human plasma [Fig. 4(b)] and protein-free human plasma [Fig. 5(b)] spiked with etoposide, etoposide catechol and internal standard.

Etoposide catechol eluted at 4.2 min, etoposide at 5.7 min and the internal standard at 8.6 min. All three compounds were resolved completely with acceptable peak shape. The SRM response of teniposide was approximately twice that of etoposide under the conditions of the assay. This resulted in a peak area ratio of ~0.5 when equal amounts were injected on-column. The peak width for etoposide was significantly greater than that for teniposide so that the peak height of etoposide was considerably lower than that of teniposide (Figs 4 and 5).

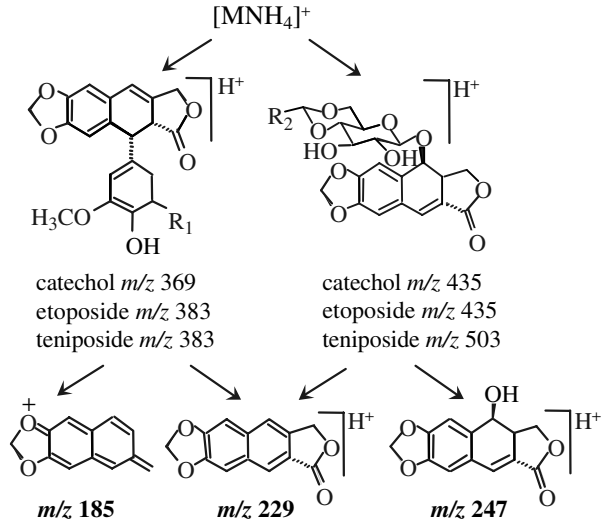


Figure 3. Proposed fragmentation pathways of etoposide, etoposide catechol and teniposide.

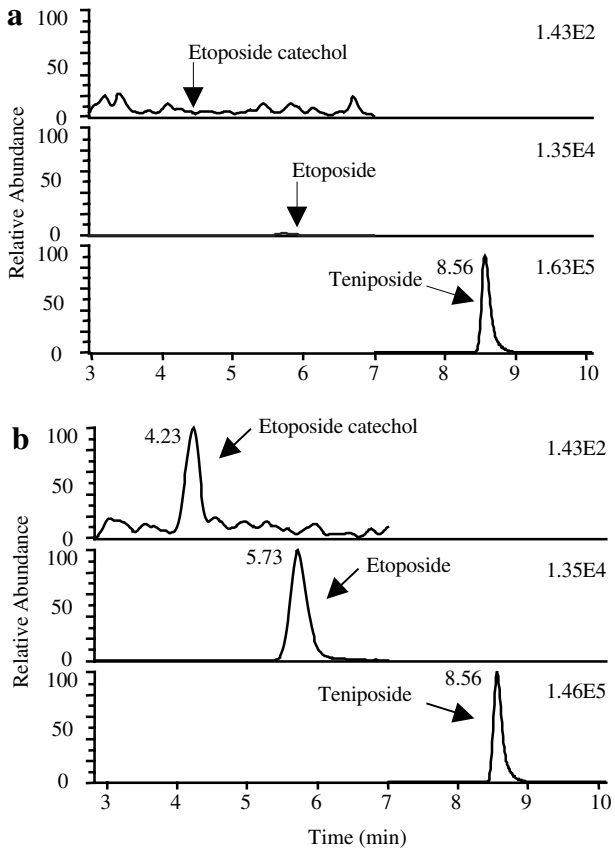


Figure 4. Representative SRM chromatograms of (a) blank human plasma spiked with teniposide (internal standard, 0.5 μg ml^{-1}) and (b) blank human plasma spiked with etoposide catechol (25 ng ml^{-1}), etoposide (0.5 μg ml^{-1}) and teniposide (0.5 μg ml^{-1}). Positive ESI was performed in the SRM mode with transitions of *m/z* 606 → 229 for etoposide, *m/z* 592 → 229 for etoposide catechol and *m/z* 674 → 383 for teniposide (internal standard). Argon at 3.5 mTorr was used as the collision gas. The collision energy was set at −32 eV for both etoposide and teniposide and at −25 eV for etoposide catechol.

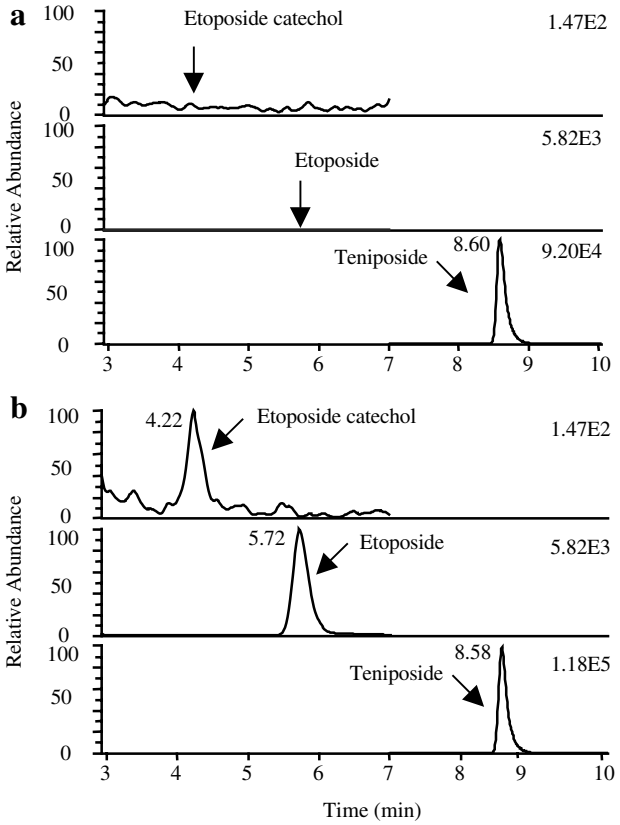


Figure 5. Representative SRM chromatograms of (a) blank protein-free plasma spiked with teniposide (0.5 μg ml^{-1}) and (b) blank protein-free plasma spiked with etoposide catechol (10 ng ml^{-1}), etoposide (100 ng ml^{-1}) and teniposide (internal standard, 0.5 μg ml^{-1}). SRM conditions as in Fig. 4.

Calibration curves

The calibration curves were plots of peak area ratios of analyte/internal standard versus the analyte concentration. The concentration of the standards ranged from 0.2 to 100 μg ml^{-1} for total etoposide, from 25 to 15 000 ng ml^{-1} for protein-free etoposide, from 10 to 5000 ng ml^{-1} for total etoposide catechol and from 2.5 to 1500 ng ml^{-1} for protein-free etoposide catechol. The means, standard deviations and RSDs were reproducible and demonstrated linearity for all of the three days of the assay validation. In order to achieve the best linearity, the data points were fitted to a linear least-squares regression curve using the weighting factors $1/x^2$, $1/x$ or a simple linear regression. The weighting factor $1/x^2$ was found to provide the best fit as determined by the r^2 value and so this was used for all curve fitting. The calibration curves were plotted with the peak area ratio of the analyte to internal standard on the *y*-axis and the concentration of the analyte on the x-axis. Typical regression line equations and correction coefficients (r^2) were as follows: $y = 0.0129 + 0.738x$ ($r^2 = 0.999$) for total etoposide (concentrations in μg ml^{-1}) and $y = 0.00445 + 0.00375x$ ($r^2 = 0.998$) for protein-free etoposide (concentrations in ng ml^{-1}). A typical regression line for total etoposide catechol (concentrations in ng ml^{-1}) was $y = 0.000342 + 0.000147x$ ($r^2 = 0.997$) and for protein-free etoposide catechol (concentrations in ng ml^{-1}) it was $y = 0.000593 + 0.000668x$ ($r^2 = 0.997$).

Stability of etoposide catechol

Etoposide catechol is readily oxidized to the corresponding quinone in the presence of oxidants, such as oxygen in the air. It has been reported that ascorbic acid could protect etoposide catechol from oxidation in the air.[24] In this study we examined the stability of etoposide catechol at different concentrations over three days in the presence of various concentrations of ascorbic acid. As shown in Fig. 6, in the absence of ascorbic acid, etoposide catechol was quickly oxidized to the quinone. In the presence of lower concentrations of ascorbic acid (0.2 and 2 mM), etoposide catechol was relatively stable, but only within 2–3 h. In the presence of 50 mM ascorbic acid, both samples containing 10 and 1000 ng ml^{-1} etoposide catechol showed a constant area ratio for the three-day testing period. Therefore, 50 mM ascorbic acid was added to the plasma samples to protect etoposide catechol from oxidation. The stability of etoposide

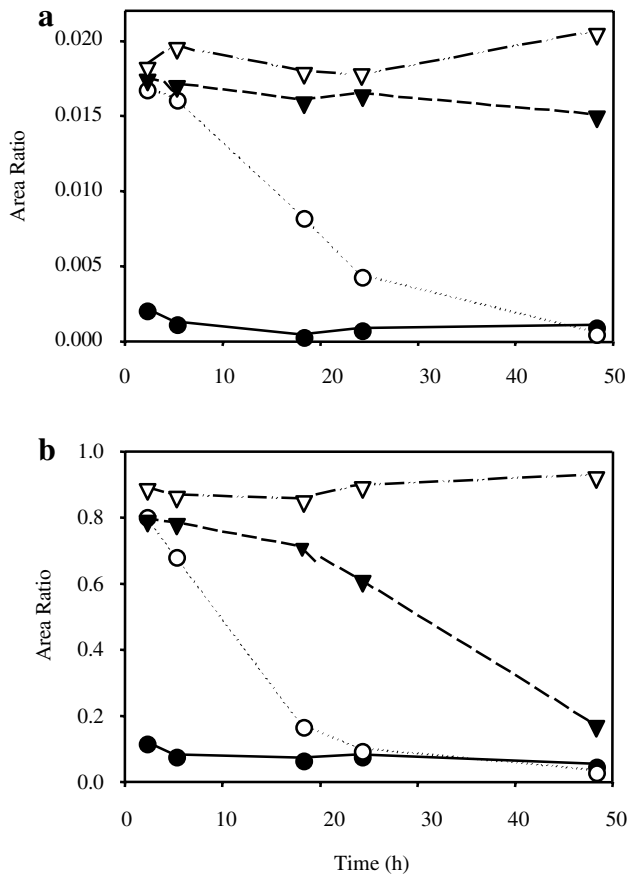


Figure 6. Etoposide catechol metabolite stability in the presence of different concentrations of ascorbic acid as antioxidant. Samples were prepared in duplicate with two concentrations of etoposide catechol in plasma with different concentration of ascorbic acid. (●) 0.0 mM ascorbic acid; (○) 0.2 mM ascorbic acid; (▼) 2 mM ascorbic acid; (▽) 50 mM ascorbic acid. (a) 10 and (b) 1000 ng ml^{-1} etoposide catechol. Area ratio is the peak area of the etoposide catechol to the internal standard, teniposide.

catechol in the plasma at room temperature was also tested. The plasma samples were left at room temperature and the change in etoposide catechol concentration was monitored. As shown in Fig. 7, etoposide catechol was stable for more than 4 h, which was long enough to prepare the plasma samples. The results also indicated that etoposide catechol was stable in plasma and that no etoposide catechol was lost during the blood sample treatment.

Reduction of etoposide quinone to catechol by ascorbic acid

It has been reported that etoposide catechol readily undergoes a two-electron oxidation to form etoposide quinone.[3,4] Attempts were made to determine the quinone in the presence of etoposide and etoposide catechol. However, etoposide quinone proved to be too unstable for reliable assay methodology to be developed. It was also reduced to etoposide catechol in the electrospray ion source during LC/ESI-MS analysis. Therefore, any quinone that was present in the plasma was first reduced to the catechol using ascorbic acid[4] and it was not included as an analyte in this study. The ascorbic acid also protected etoposide catechol against adventitious oxidation during the work-up and analysis procedure.

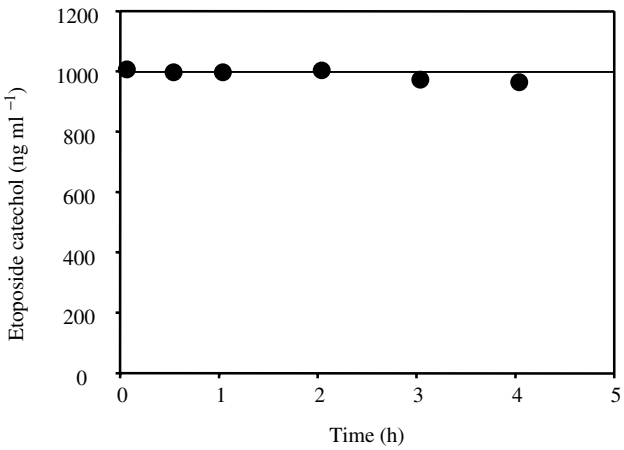


Figure 7. Etoposide catechol stability in plasma at room temperature. Etoposide catechol was spiked at 1000 ng ml^{-1} into duplicate plasma samples and left at room temperature. Samples were taken at different time points and analyzed by LC/MS. The horizontal line represents the starting concentration of 1000 ng ml^{-1}.

Precision and accuracy

The lower limits of quantification (LLQ) of total and protein-free etoposide catechol were determined as 10 and 2.5 ng ml^{-1}, respectively. These were the lowest concentrations that could be measured with a precision of ±20% and an accuracy between 80 and 120% ($n = 5$), the criteria established by Shah *et al*.[30] In order to quantify etoposide and etoposide catechol simultaneously, the ratio of the two analytes was set at 20 : 1 for total and 10 : 1 for the protein-free fraction. Therefore, the LLQs for total and protein-free etoposide were 200 and 25 ng ml^{-1}, respectively. The determined mean of LLQs of total etoposide and protein-free etoposide were 0.216 μg ml^{-1} and 27.07 ng ml^{-1}, respectively (Table 1). For total and protein-free etoposide catechol the LLQs were determined as 10.38 and 2.49 ng ml^{-1}, respectively (Table 2). The intra-day precision of the LQC, MQC and HQC ranged from 1.3 to 6.7% for total and protein-free etoposide, respectively, and from 0.4 to 5.8% for total and protein-free etoposide catechol, respectively (data not shown). The inter-day precision for total and protein-free etoposide for the LQC, MQC and HQC ranged from 0.7 to 3.2% and the accuracy (observed mean/theoretical × 100) was between 94 and 108% for all QC samples (Table 1). The inter-day precision for total and protein-free etoposide catechol for the LQC, MQC and HQC ranged from 1.0 to 5.2% and the accuracy was better than 96–104% for all QC samples (Table 2). The recommended[30] intra- and inter-day precision (RSD 15%) and accuracy (85–115%) requirements for the LQC, MQC and HQC samples were met for all analytes. Therefore, the method was sensitive, accurate and highly reproducible for the determination of total and protein-free etoposide and etoposide catechol in human plasma within a wide dynamic range.

Table 1. Precision and accuracy for the determination of total and protein-free etoposide in human plasma

Compound	Parameter	LLQ	LQC	MQC	HQC
Total etoposide	Theoretical (μg ml^{-1})	0.200	0.500	5.00	50.0
	n	5	15	15	15
	Mean (μg ml^{-1})	0.216	0.505	4.97	51.5
	RSD (%)	2.4	1.9	0.7	3.2
	Accuracy (%)	108	101	99.3	103
Free etoposide	Theoretical (ng ml^{-1})	25.00	100.0	1000	10 000
	n	5	15	15	15
	Mean (ng ml^{-1})	27.07	103.1	1006	9433
	RSD (%)	1.9	1.3	2.5	2.4
	Accuracy (%)	108	103	101	94.3

Table 2. Precision and accuracy for the determination of total and protein-free etoposide catechol in human plasma

Compound	Parameter	LLQ	LQC	MQC	HQC
Total catechol	Theoretical (ng ml^{-1})	10.00	25.00	250.0	2500
	n	5	15	15	15
	Mean (ng ml^{-1})	10.38	24.87	249.1	2417
	RSD (%)	4.3	1.2	3.5	5.2
	Accuracy (%)	104	99.5	99.7	96.7
Free catechol	Theoretical (ng ml^{-1})	2.50	10.00	100.0	1000
	n	5	15	15	15
	Mean (ng ml^{-1})	2.49	10.03	101.9	973
	RSD (%)	6.5	1.0	1.6	2.1
	Accuracy (%)	99.5	100	102	97.4

Sample stability and recovery

The processed standard samples were stable over 24 h when left in the HPLC autosampler at 5 °C (data not shown). In order to determine the recovery, plasma and protein-free plasma samples at MQC concentrations were processed as described in the Experimental section and compared with LC/MS analyses from the processed plasma blanks that were spiked with standards at the MQC concentrations. The recoveries for etoposide and etoposide catechol were 78.9 and 91.3%, respectively, from human plasma ($n = 3$) and 99.0 and 101%, respectively, from protein-free plasma ($n = 3$). To determine the plasma sample matrix effect on analyte ionization, blank plasma and protein-free plasma samples were processed as described in the Experimental section, spiked at the MQC concentrations and compared with LC/MS analyses of aqueous unextracted standards. The suppression of ionization for etoposide and etoposide catechol was 3.3 and 5.3%, respectively, for human plasma ($n = 3$) and 4.4 and 12.5%, respectively, for plasma ultrafiltrate ($n = 3$).

Application of the method to pharmacokinetic studies

One representative pediatric cancer patient received a 100 mg m^{-2} dose of etoposide as a 1 h infusion daily for 5 days. On days 1 and day 5, blood samples were taken at selected time points before and after completion of the infusion. The plasma was treated with ascorbic acid, which would have converted any etoposide quinone back to the catechol. Therefore, the catechol measurements would include any quinone that may have been present at the time of sample collection. The total and protein-free fraction of etoposide and etoposide catechol in the plasma were determined by LC/SRM/MS. A typical chromatogram of total etoposide and total catechol from the patient 150 min after dosing is shown in Fig. 8(a). A chromatogram showing protein-free etoposide and catechol at the same time-point is shown in Fig. 8(b).

In order to elucidate the mechanisms by which secondary leukemias occur after etoposide treatment, it is important to determine inter-patient differences in pharmacokinetic parameters. This required the development of a rational pharmacokinetic model. Using the WinSAAM modeling program we obtained the best fit of the plasma concentration–time parameters with the model shown in Fig. 9.

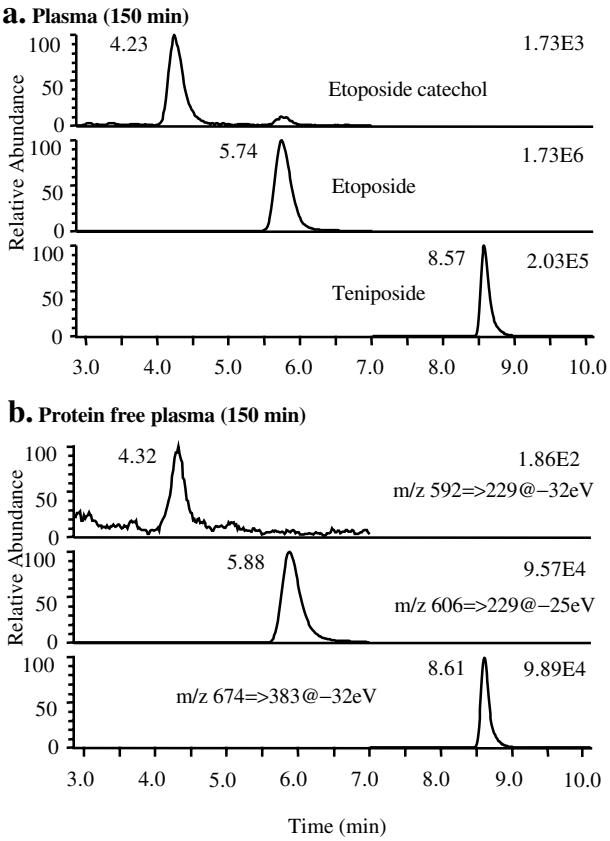


Figure 8. Representative SRM chromatograms of (a) plasma and (b) protein-free plasma samples obtained from a pediatric patient 150 min after receiving a 100 mg m^{-2} dose of etoposide as a 1 h infusion. SRM conditions as in Fig. 4.

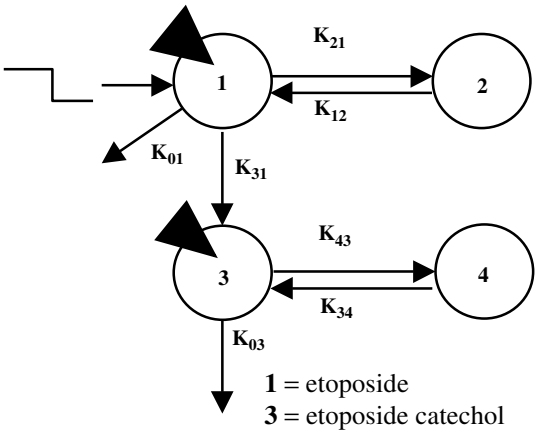


Figure 9. Pharmacokinetic model for etoposide metabolism to its catechol metabolite. The *k* values represent the fractional rates for transfer from one compartment to the next.

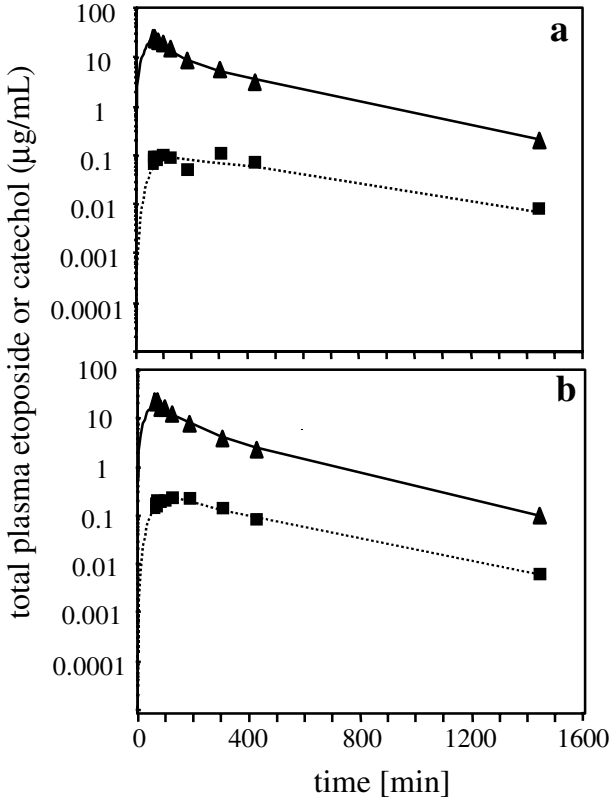


Figure 10. Plasma concentration–time profile for subject A given a 100 mg m^{-2} intravenous infusion of etoposide over 1 h. The solid triangles and solid squares are the plasma concentrations determined by LC/MS for etoposide and its catechol metabolite, respectively. The solid line represents the calculated values for etoposide concentrations and the dashed line represents the calculated values for the catechol metabolite concentrations using the kinetic model in Fig. 9. (a) Day 1; (b) day 5.

This first-order two-compartment model has the same topology as that described by Relling *et al*.[31] The model, which takes into account both elimination and metabolism, was able to fit the total plasma etoposide and etoposide catechol as well as the free etoposide and etoposide catechol

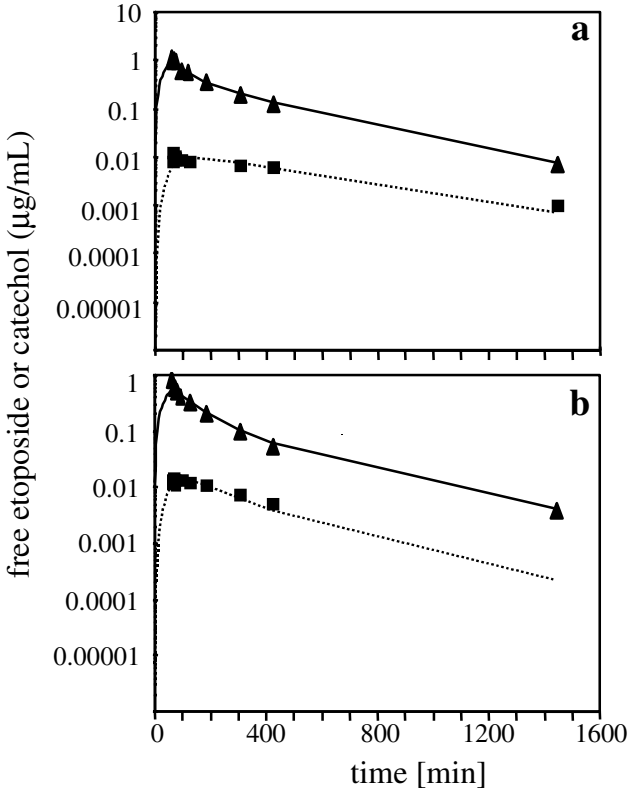


Figure 11. Plasma ultrafiltrate concentration–time profile for subject A given a 100 mg m^{-2} intravenous infusion of etoposide over 1 h. Symbols as in Fig. 10. (a) Day 1; (b) day 5.

concentrations. The distribution spaces for etoposide and its catechol metabolite were assumed to be the same, and the inter-compartmental rate constants were assumed to have the same value for the catechol movement. Unique values of the etoposide inter-compartmental rate constants were estimated from the data. To ease the fitting process, the final (elimination) phase of the etoposide was fitted first, and then the partition of that elimination between irreversible loss and conversion to the catechol was resolved in subsequent data fitting, involving both etoposide and its catechol metabolite. This made it possible for the first time to determine simultaneous pharmacokinetic parameters for total and free parent drug and also its major metabolite.

The plasma concentration–time curves for total etoposide and etoposide catechol are presented in Fig. 10. On day 1, the peak plasma concentration of total etoposide (C_{max}) was 24.02 µg ml^{-1}, which was achieved at the time point (t_{max}) of 61 min, just after the end of the infusion. For total etoposide catechol the C_{max} was 106 ng ml^{-1} and t_{max} was 90 min. The plasma concentration–time curves of protein-free etoposide and etoposide catechol are shown in Fig. 11. C_{max} for protein-free etoposide and etoposide catechol on day 1 were 1.16 and 11 ng ml^{-1}, respectively. On day 5, both total and protein-free concentrations of etoposide were similar to day 1 (Figs 10 and 11). However, the C_{max} for total (217 ng ml^{-1}) and protein-free (14 ng ml^{-1}) etoposide catechol were significantly higher on day 5 when compared with day 1 (106, 11 ng ml^{-1}) (Table 4). From our WinSAAM

Table 3. Pharmacokinetic parameters for total and protein-free etoposide from a patient dosed with 100 mg m^{-2} of etoposide on days 1 and 5

Parameter	Total etoposide day 1	Free etoposide day 1	Total etoposide day 5	Free etoposide day 5
C_{max} (μg ml^{-1})	24.02	1.16	23.37	0.81
Volume of distribution (ml)	4086	102	5052	185
$t_{1/2}\alpha$ (min)	41	49	64	75
$t_{1/2}\beta$ (min)	255	249	237	301
AUC_{6h} (μg ml^{-1} min)	3496	145	3165	86
AUC_{24h} (μg ml^{-1} min)	4957	198	4043	109
Mean residence time (min)	295	279	247	249

Table 4. Pharmacokinetic parameters for total and protein-free etoposide catechol from a patient dosed with 100 mg m^{-2} of etoposide on days 1 and 5

Parameter	Total catechol day 1	Free catechol day 1	Total catechol day 5	Free catechol day 5
C_{max} (ng ml^{-1})	106	11	217	14
$t_{1/2}\alpha$ (min)	24	35	24	24
$t_{1/2}\beta$ (min)	193	ND[a]	205	ND
AUC_{6h} (μg ml^{-1} min)	25.0	2.8	56.7	3.3
AUC_{24h} (μg ml^{-1} min)	56.8	ND	93.0	ND

[a] ND = not determined because concentrations were too low.

model (Fig. 9), the major pharmacokinetic parameters for total and protein-free etoposide and etoposide catechol were determined for an etoposide infusion of 100 mg m^{-2} for 1 h (Tables 3 and 4). The initial (distribution) half-lives ($t_{1/2}\alpha$) for total etoposide following infusion of etoposide were 41 and 64 min for days 1 and 5, respectively. This result was in agreement with those reported previously.[1,31,32] The $t_{1/2}\alpha$ values for protein-free etoposide were similar to those for total etoposide (Table 3). The $t_{1/2}\alpha$ values for total and protein-free etoposide catechol were slightly shorter than those for the parent drug (Table 4). The terminal phase elimination half-lives ($t_{1/2}\beta$) for total etoposide were 255 and 237 min for days 1 and 5, respectively, which was within the range of 3.4–8.1 h reported previously.[1,31,32] The $t_{1/2}\beta$ values for total etoposide catechol were slightly longer than those for the parent drug (Table 4). The AUC_{24h} for total etoposide was significantly lower on day 5 compared with day 1 (Table 3). Interestingly, the AUC_{24h} (93 μg ml^{-1} min) for total etoposide catechol on day 5 was almost double that for day 1 (57 μg ml^{-1}min) (Table 4). This trend was also observed for the protein-free catechol, although the low protein-free concentrations at 1440 min precluded a full pharmacokinetic analysis. This suggested that the potentially toxic catechol metabolite could accumulate during multiple-day dosing. Additional studies in more patients will be required in order to confirm this observation. However, it is noteworthy that a previous study reported the accumulation of etoposide catechol during etoposide therapy.[33] Our pharmacokinetic model is currently being used to correlate inter-patient differences in etoposide and etoposide catechol pharmacokinetics with CYP3A4 genotype.[15] This information will ultimately be useful for optimizing etoposide dose and schedule so that the risk of secondary leukemia is minimized.

CONCLUSIONS

We have developed and validated a sensitive and selective LC/SRM/MS method for the determination of both total and protein-free etoposide and its potentially toxic catechol metabolite in human plasma. This method was able to quantify etoposide and etoposide catechol simultaneously with a concentration ratio up to 300 or higher in the plasma of pediatric cancer patients. Good linearity, precision and accuracy were achieved. The limit of quantitation was 10 ng ml^{-1} for total etoposide catechol and 2.5 ng ml^{-1} for the protein-free fraction. For total concentrations, simultaneous determination of etoposide and its catechol metabolite was possible with only 50 μl of plasma. With simple sample treatment and small sample requirements, this method has been applied successfully to pharmacokinetic studies of pediatric cancer patient receiving intravenous etoposide. In addition, we developed a pharmacokinetic model for a future pharmacogenetic study to determine if there is a correlation between phenotype and genotype.[15] The full pharmacokinetic studies will provide additional insight into the mechanisms by which secondary leukemias may occur during etoposide treatment. Finally, the LC/MS methodology may also make it possible to optimize etoposide dose and schedule in order to minimize the risk of leukemogenesis.

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