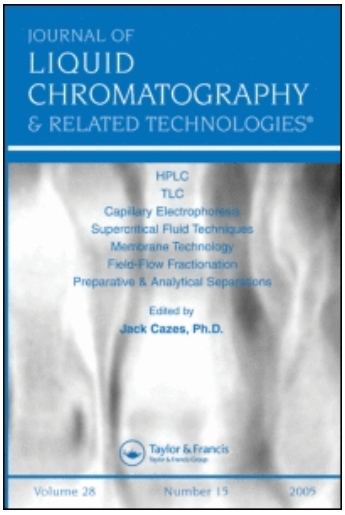

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Fleming, Ronald A. and Stewart, Clinton F.(1991) 'High-Performance Liquid Chromatographic Determination of Etoposide in Plasma', Journal of Liquid Chromatography & Related Technologies, 14: 7, 1275 — 1283
To link to this Article: DOI: 10.1080/01483919108049320
URL: http://dx.doi.org/10.1080/01483919108049320

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(7), 1275-1283 (1991)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ETOPOSIDE IN PLASMA

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ABSTRACT

A rapid and sensitive high-performance liquid chromatographic method for the determination of etoposide in plasma is described. Etoposide and the internal standard phenacetin were extracted with 1,2 dichloroethane. Separation was achieved using a phenyl (300 x 3.9 mm, 5 μm packing) analytical column. The effluent was monitored by UV detection at a wavelength of 233 nm. The etoposide peak-height ratio to the internal standard was linearly related to etoposide plasma concentration over a range of 0.1 - 50 μg/mL ($r^2 = 0.998$). Reliability of the assay was excellent with both the within- and between- day coefficient of variation $< 5\%$. We have observed this method to be both sensitive and reliable, qualities important in our recent application of this method in a high-dose etoposide pharmacokinetic study in cancer patients.

INTRODUCTION

Etoposide (VP-16), a semisynthetic derivative of podophyllotoxin, is used in combination with other antineoplastic agents in treating small cell lung cancer, testicular cancer, ovarian cancer, and leukemia.(1) Recent studies (2-4) have suggested that hematologic toxicity (leukopenia, thrombocytopenia) following etoposide administration is related to plasma etoposide systemic exposure. Thus, interpatient pharmacokinetic variability may contribute to the variability in toxicities attributed to etoposide. Ratain and associates (5) have attempted to reduce the intra-and interpatient pharmacokinetic variability of etoposide through the use of adaptive control methodology, whereby etoposide dose is adjusted based upon serum concentrations.

For pharmacologically guided adjustments of etoposide dosage to be successfully introduced into clinical oncology practice, a sensitive, rapid method for determining etoposide plasma concentrations is necessary. Several high-performance chromatographic methods for determining etoposide plasma concentrations using UV (6-8), fluorescence (9), and electrochemical (10) detection have been published. Some of these methods are limited by lengthy extraction methods (8), require large volumes of plasma (2 ml) (6), or have long retention times.(10) In this paper, a simple, sensitive HPLC method with UV detection for determining etoposide plasma concentrations is described.

MATERIALS

Instrumentation

The HPLC system consisted of a Beckman (Fullerton, CA) 110B Solvent Delivery Module, an LDC/Milton Roy spectroMonitor III variable wavelength UV detector (Riveria Beach, FL) operated at 233 nm, a Spectra-Physics (San Jose, CA) SP8780 autosampler, and a Rheodyne (Cotati, CA) 7125 injector. A precolumn (100 X 3.4 mm) packed with C_{18} bonded silica (10 μm particle size) was placed between the injector and the analytical column. Separation was achieved using a phenyl (Waters μbondapak) column 300 x 3.9 mm with 5 μm packing (Waters, Bedford, Mass.). All separations were performed at a flow rate of 1 mL/min with column temperatures at 24 to 27°C (ambient

temperature). Detection outputs were computed with a Shimadzu (Columbia, MD) C-R5A Chromatopac integrator. Detection signal was attenuated by a factor of 64 (2^6).

Chemicals and Reagents

Etoposide was kindly provided by the Analytical Research and Development Department, Bristol-Myers Company - Industrial Division (Syracuse, NY). Phenacetin (used as the internal standard) was obtained from the Sigma Chemical Company (St. Louis, MO). Analytical grade acetonitrile, methanol, acetic acid, and 1,2 dichloroethane were obtained from Fisher Chemical Company (St. Louis, MO). All water used in the HPLC system was distilled and deionized (Milli-Q System, Millipore Corp., Bedford, MA).

Mobile Phase

The mobile phase which consisted of a 70:30:1 mixture of deionized and distilled water, acetonitrile, and acetic acid, was filtered and degassed with nitrogen for 10 minutes prior to use.

METHODS

Extraction Procedure

The patient sample, plasma standard, or control (500 μl) was pipetted into a test tube along with 50 μl of the phenacetin internal standard solution and the mixture was vortexed for 30 seconds. Then, 3 mL of 1,2 dichloroethane were added, and the mixture vortexed vigorously for 1 minute. The tube was centrifuged at 1500 G for 5 minutes, and the lower organic layer was transferred into a small test tube, evaporated under nitrogen, and finally reconstituted with 150 μl of a 70:30 acetonitrile and deionized water mixture. The solution was vortexed for 30 seconds, transferred to an autosampler vial, and 100 μl of the solution was injected onto the HPLC system.

Extraction efficiency was determined by comparing the etoposide peak area in extracted etoposide controls of 0.55, 7.5, 15, and 45 μg/ml with blank plasma samples which were extracted, and then spiked with etoposide at the same concentrations as the

plasma controls. Five samples at each concentration were analyzed for both the extracted and unextracted samples.

Calibration Procedure

A 1 mg/mL stock solution of etoposide in methanol was prepared along with 1:10 and 1:100 dilutions of the stock (with methanol) to ensure that the volume of methanol in etoposide standards or controls was < 8% of the total volume. In addition, a 5 μg/mL solution of phenacetin in methanol was prepared as the internal standard solution. Appropriate amounts of the etoposide stock solution were added to pooled donor plasma to prepare standards of 0.1, 0.5, 1, 2.5, 10, 20, 30, 40, and 50 μg/mL. Etoposide controls of 0.55, 7.5, 15, and 45 μg/mL were prepared in a similar manner.

Linearity studies

A standard curve was prepared with duplicate etoposide standards at each concentration (see above) to assess the linearity between the etoposide concentration and the peak-height ratios of etoposide to the internal standard phenacetin.

Precision and accuracy studies

To assess both within- and between-day variability of the assay, on days 1-5, five controls at each concentration (0.55, 7.5, 15, and 45 μg/ml) were analyzed. Etoposide concentrations for the controls were calculated from the standard curve run on day 1. Both within-and between-day percent coefficient of variation (%CV) were calculated for each etoposide control concentration.

Application of Method

Patients with advanced lymphoma refractory to conventional therapy were enrolled onto an experimental protocol evaluating the role of autologous bone marrow transplantation. As part of the preparative regimen, patients received high dose oral busulfan (4 mg/kg) followed by high dose intravenous etoposide (50-60 mg/kg). Etoposide pharmacokinetics were evaluated, and depicted in figure 1 is an etoposide concentration versus time plot for a representative patient.

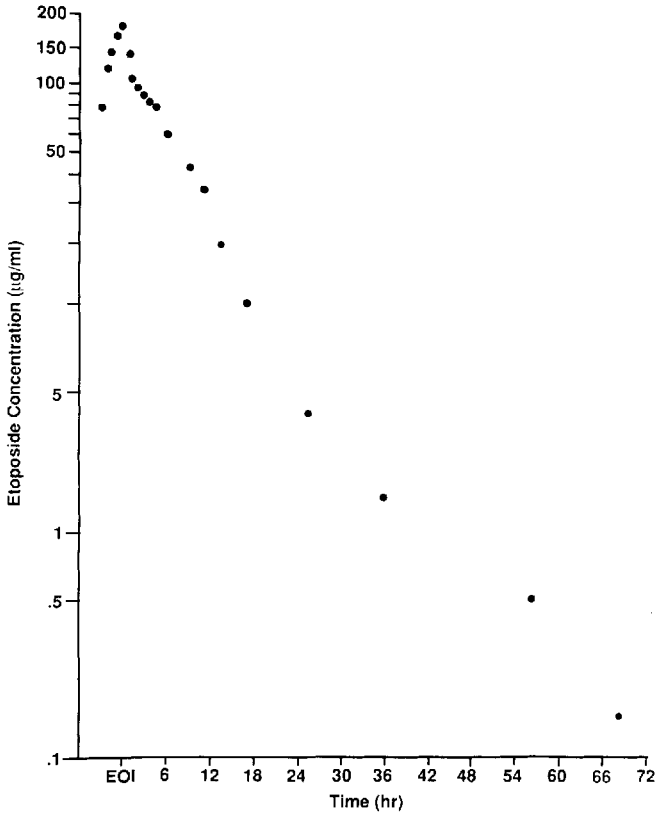

Figure 1: Concentration versus time plot for a representative patient receiving high dose etoposide (50 mg/kg).

RESULTS AND DISCUSSION

Typical chromatograms of blank plasma (Panel A) and plasma with phenacetin (7.4 minutes) and etoposide (9.9 minutes) (Panel B) are shown in Figure 2. A linear relationship was observed between etoposide concentration and the ratio of etoposide peak height to phenactin peak height from 0.1 to 50 μg/ml ($y = 1.83 * - 0.053$; $r^2 = 0.998$). The lower limit of detection for etoposide was 0.05 μg/ml. The lower limit of detection

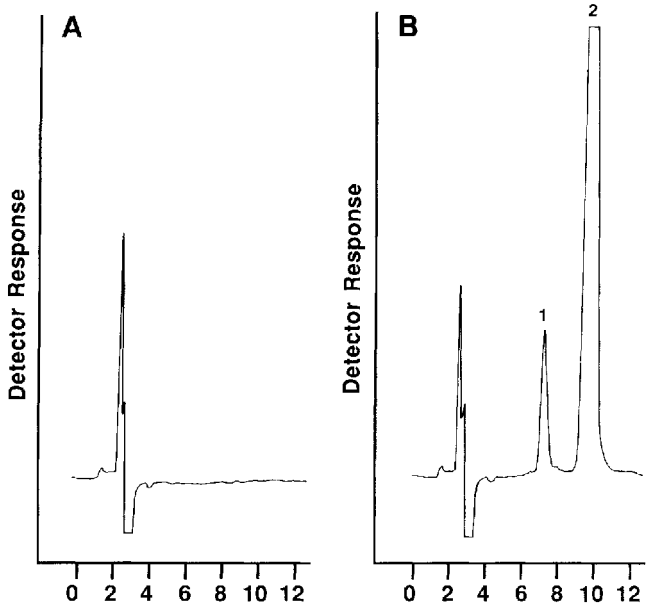

Figure 2: Chromatogram of extracted blank plasma (Panel A) and of human plasma spiked with the internal standard phenacetin (retention time 7.4 minutes) and etoposide 7.5 μg/ml (retention time 9.9 minutes).

Table 1

Within-day Precision and Accuracy for Etoposide Controls

Actual Etoposide Conc. (μg/ml)	Calculated Etoposide Conc. (μg/ml)	%C.V.[1]	% Error[2]
0.55	0.518	4.8	5.8
7.5	7.22	1.3	3.7
15	14.36	0.8	4.3
45	41.93	2.0	6.8

[1] Precision expressed as %CV for five etoposide controls at each concentration.

[2] $\frac{\text{Actual Etoposide concentration - Calc. Etoposide Conc.}}{\text{Actual etoposide concentration}} \times 100\%$

Table 2

Between-day Precision and Accuracy for Etoposide Controls

Actual Etoposide Conc. (μg/ml)	Calculated Etoposide Conc. (μg/ml)	%C.V.[1]	% Error[2]
0.55	0.53	4.9	3.6
7.5	7.60	3.5	1.3
15	15.46	4.0	3.1
45	43.85	4.8	2.6

[1] Precision expressed as %CV for the mean etoposide calculated concentration for days 1-5.

[2] $\frac{\text{Actual Etoposide concentration - Calc. Etoposide Conc.}}{\text{Actual etoposide concentration}} \times 100\%$

was defined as that concentration of etoposide which gave a signal at least three times that of background noise.

As seen in Table 1, the within-day accuracy and precision for the four etoposide controls were excellent. Similarly, the assay was shown to be both accurate and precise over 5 consecutive days (Table 2).

The mean extraction efficiencies (%CV) for the 0.55, 7.5, 15, and 45 μg/ml etoposide controls were 96% (2.1%), 95.8% (1.7%), 95.5% (1.5%), and 95.2% (1.8%) respectively.

As knowledge of the clinical pharmacodynamics of etoposide increases relation between etoposide concentration in plasma and either toxicity or antitumor response, it is essential that a simple, reliable method for determining etoposide concentrations be made available. Of the many methods of detection electrochemical detection may be more sensitive than UV detection in determining etoposide concentration; however, characteristics of the UV method of detection (i.e., stability of instrument. lack of prolonged equilibration before use) (7) make it more practical to use in the clinical setting than electrochemical detection.

The assay described in this report which utilizes UV detection is sensitive, precise, and accurate, qualities necessary for a clinical assay which would be used for routine monitoring of etoposide serum concentrations.

AKNOWLEDGEMENTS

The provision of plasma etoposide samples for use in this study by Dr. Terry Schwinghammner is gratefully acknowledged.

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