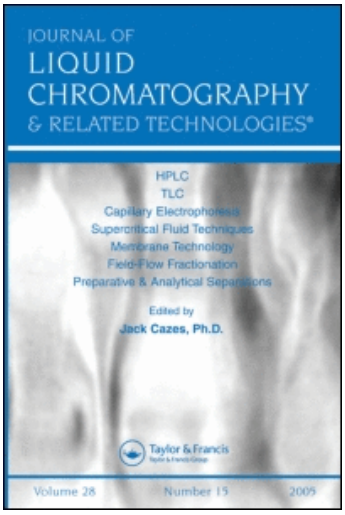

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ETOPOSIDE IN PLASMA USING FLUORESCENCE DETECTION

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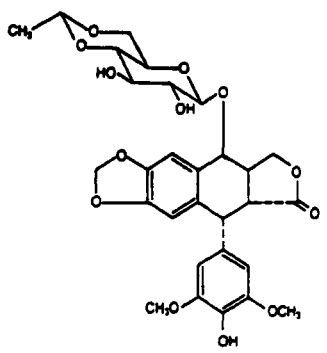
ABSTRACT

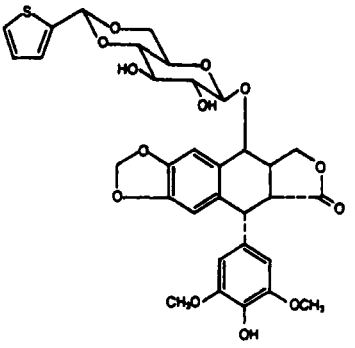
A rapid and sensitive high-performance liquid chromatographic assay was developed for the quantitation of the antineoplastic drug etoposide in plasma. Etoposide and the internal standard teniposide were extracted from plasma (1ml) with methylene chloride. The extracts were analyzed on an Alltech C18 Econosil column with a mobile phase of 40% methanol, 15% acetonitrile, 44% water, and 1% acetic acid. The assay utilized fluorescence detection (EX: 230 nm and EM: 328 nm) which provided sensitivity and specificity sufficient to quantify $\geq$ 50 ng/ml etoposide in plasma. The correlation coefficient of the multilevel standard curve was = 0.999 over a concentration range of 50-1000 ng/ml. The interday and intraday coefficients of variation were < 7%. This method has been used by our laboratory to provide the unattended overnight analysis of etoposide in plasma obtained from bone marrow transplant patients.

INTRODUCTION

Etoposide (VP-16-213) is an antineoplastic agent that in conventional doses (250-500 mg/m^2) has activity in small cell lung cancer, testicular cancer, and other diseases (1). High dose etoposide (up to 2.4 g/m^2 over 3 days or 1-3 g/m^2 as a single 4-hour infusion) has recently been used as a component of preparative regimens prior to autologous bone marrow transplantation (ABMT) (2). In the setting of ABMT, infusion of healthy marrow should be delayed until post-dose etoposide plasma concentrations have declined to a level which is not toxic to bone marrow progenitor cells. Studies in our laboratory and elsewhere have suggested that plasma etoposide concentrations of < 400 ng/ml are unlikely to result in delayed engraftment (3,4). However, interpatient pharmacokinetic variability makes it difficult to predict the time required for etoposide concentrations to decrease to those levels. As a result, measurement of plasma etoposide prior to ABMT is useful to identify those patients with reduced clearance in whom delay of transplantation may be advisable. Because of the wide range of etoposide concentrations observed after high doses, a sensitive and specific assay for etoposide is required for this purpose. Several liquid chromatographic methods for the analysis of etoposide (Figure 1) in biological fluids have been described. The modes of detection most commonly used for this assay are ultraviolet and electrochemical (5-10). However, due to the limited sensitivity of ultraviolet detection and the instability coupled with extensive equilibration associated with electrochemical detection, the use of a third method of detection, fluorescence, has been employed. Fluorescence detection methods described in the literature to date demonstrate sensitivities at or near that obtained with electrochemical detection (11,12). The major drawbacks to these methods, however, are the need for complex multi-component instrumentation or the requirement of extended run times due to the appearance of fluorescing substances at long retention times which may interfere with subsequent injections. This paper describes a simple, economical, specific, and highly sensitive HPLC method using fluorescence detection for the quantitation of etoposide in plasma. This method is suitable for pharmacokinetic and drug level monitoring of etoposide in patients.



Etoposide



Teniposide

FIGURE 1: Etoposide and the internal standard, teniposide.

MATERIALS

Reagents and Chemicals

Etoposide and teniposide were generously provided by the Pharmaceutical Research Institute of the Bristol-Myers Squibb Co. (Evansville, IN). Methanol, acetonitrile, glacial acetic acid, and methylene chloride were of HPLC grade and obtained from Fisher Scientific Co. (Pittsburgh, PA). Sodium acetate was of analytical grade and obtained from Aldrich Chemical Co. (Milwaukee, WI). All water used in the analysis and HPLC mobile phase was deionized and distilled with a Milli-Q Reagent Water System (Millipore Corp., Bedford, MA). Blank plasma used for the preparation of standards and quality control samples was obtained from in-house donors as well as through the local blood bank. Extraction of plasma was carried out in 16 x 125 mm glass culture tubes with PTFE-lined caps. Polypropylene sample inserts used in the HPLC sample processor were obtained from Sun Brokers Inc. (Wilmington, NC).

Instrumentation

The HPLC system consisted of a Waters Associates model 510 solvent delivery pump, model 712 Wisp automatic sample processor, and model 470

scanning fluorescence detector. The detector was operated at an excitation wavelength of 230nm and an emission wavelength of 328nm. Signal output was computed with a Hewlett-Packard (Santa Clara, CA) model 3354A integrator. Separation of the compounds of interest was achieved with an Alltech Associates (Deerfield IL) direct connect guard column (20 x 2 mm) filled with pellicular C18 packing connected to an Alltech Associates 4.6 x 250 mm Econosil 10$_\mu$ C18 analytical column. The mobile phase used for the analysis consisted of a 39:15:45:1 mixture of methanol:acetonitrile:water:acetic acid (pH 3.0) and was pumped at a flow rate of 1ml/min (1100 p.s.i.). The mixture was degassed and filtered through a 0.22 μm Nylon 66 membrane before use.

METHODS

Preparation of Stock Solution and Spiked Plasmas

A 0.1mg/ml etoposide stock solution was prepared by dissolving 10mg of etoposide in 100ml of methanol. Working solutions of 40, 20, and 5ng/μl were made by subsequent serial dilution of the stock solution. The internal standard teniposide was prepared as a 0.1mg/ml solution in methanol. These solutions were stable for 6 months when stored at 0° C. No more than 30 μl of methanol was added to plasma in the preparation of standards or quality control samples. Five hundred nanograms of teniposide was used as internal standard in 1ml plasma samples containing up to 1000ng/ml etoposide.

Extraction of Plasma Samples

To 1.0 ml of plasma in a culture tube 5 μl (500ng) of the internal standard teniposide was added. The sample was vortexed for 30 sec after which 3 ml of methylene chloride was added. The samples were shaken for 15 min (85 cycles per min) and centrifuged at 20 °C for 10 min at 2500 rpm. The upper plasma layer was aspirated and discarded. Approximately 2.5ml of the organic layer was transferred to a 5 ml conical centrifuge tube and evaporated to dryness at 40 °C under a stream of nitrogen. The samples were reconstituted with 200 μl of a solvent system similar to that used for the HPLC

mobile phase with the water/acetic acid of the mobile phase replaced with a .01M pH 4.5 sodium acetate buffer. An 80 μl aliquot was injected into the HPLC. Sodium acetate buffer was used instead of acetic acid to avoid the potential breakdown of etoposide, particularly for samples which would sit for several hours in the autosampler before injection. Etoposide is most stable in the pH range of 4-5 (13). Etoposide is subject to degradation in acidic media via cleavage of the glucopyranosyl moiety to give the aglycone. The aglycone may further degrade to the hydroxy acid derivative by hydrolysis of the cyclic ester bond of the aglycone (13).

Calibration and Linearity

Calibration curves using six different concentrations of etoposide in plasma, ranging from 50 to 1000 ng/ml, were obtained daily for 3 days by calculating the peak height ratios of etoposide to that of the internal standard teniposide versus the respective concentrations of etoposide. The concentrations of the standards evaluated were 50, 100, 300, 500, 800, and 1000 ng/ml. All standards were run as duplicates.

Precision and Accuracy

The precision and accuracy of the assay was determined through the analysis of etoposide spiked quality control plasma samples. The concentrations of the controls analyzed were 100, 500, and 900 ng/ml. For analysis days 1-4, the inter- and intra-day means, standard deviations, and percent coefficients of variation (CV) were calculated for each etoposide quality control sample.

Recovery

Recovery of etoposide was determined by comparing the peak height ratios (etoposide/teniposide) of the extracted quality control samples with the peak height ratios of unextracted standard solutions prepared in the same concentrations as the QC samples. The internal standard teniposide was added to all samples just prior to analysis.

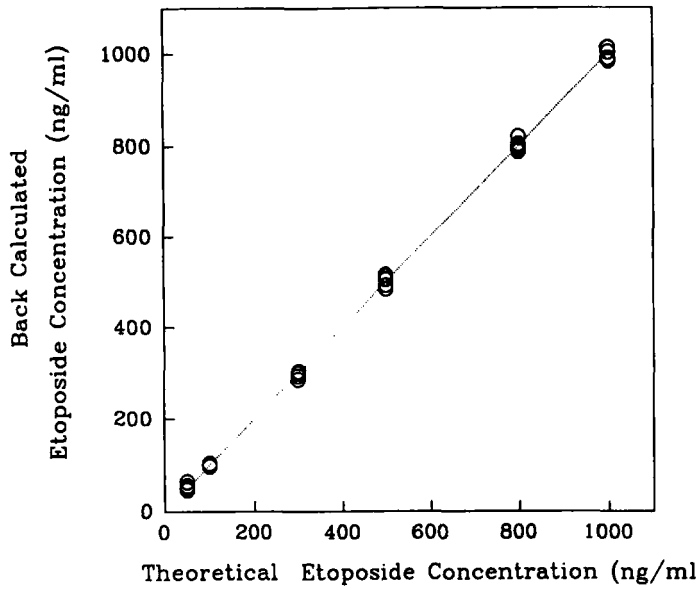


FIGURE 2: Six point calibration curves (n = 3) for etoposide in plasma which demonstrate linearity over a concentration range of 50-1000 ng/ml.

Stability

Plasma samples spiked with etoposide at 100, 500, and 900 ng/ml were stored at -80 °C for up to six months. The samples were periodically analyzed for their etoposide content over this time period.

RESULTS

Standard curves (n = 3) calculated from duplicate spiked plasma samples (50-1000 ng/ml etoposide) are shown in Figure 2. Least squares regression analysis gave a mean linear correlation coefficient of r^2 = 0.999 with a y-intercept of 0.004 and a slope of 0.022 (Table 1). No weighting factor was used for the regression analysis. Figure 3 shows typical chromatograms for blank plasma, plasma spiked with etoposide (100ng/ml), and a clinical plasma

TABLE 1

Calibration Curve Data for Etoposide in Human Plasma

Spiked Concentration (ng/ml)	PK HT Ratio (Etoposide/ISTD)	Calculated Concentration (ng/ml)
0	0	0
50	0.21	45.4
100	0.46	103.3
300	1.31	304.5
500	2.12	496.0
800	3.41	801.1
1000	4.25	999.7

Intercept: 0.022
Slope: 0.004
r^2: 0.999

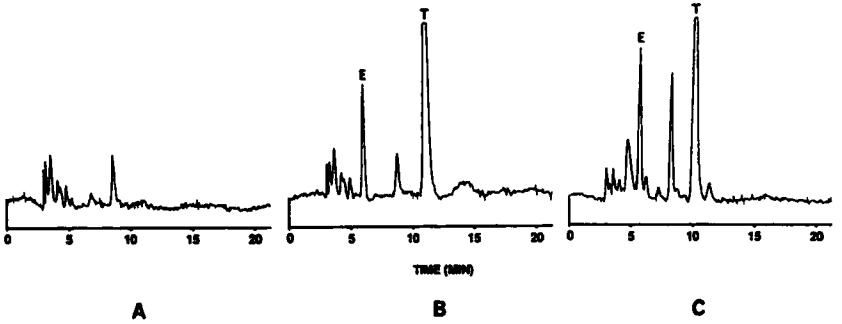


FIGURE 3: HPLC chromatograms of (A) blank plasma, (B) plasma spiked with etoposide (100ng/ml), and (C) plasma obtained from a bone marrow transplant patient 48 hours after an i.v. infusion of etoposide (300mg/m^2/day x 5 days). E = etoposide and T = teniposide.

TABLE 2

Intra-day Precision and Accuracy for Etoposide Quality Controls

Spiked Concentration:	100ng/ml	500ng/ml	900g/ml
Calculated Concentration:	97.6	464.2	871.1
	98.3	530.4	885.2
	102.2	515.8	910.2
	97.0	495.4	923.5
	91.2	463.2	909.8
	97.4	541.2	937.0
Mean:	97.3	501.7	906.1
SD:	3.5	33.2	24.3
CV%:	3.6	6.6	2.7

TABLE 3

Inter-day Precision and Accuracy for Etoposide Quality Controls

Spiked Concentration:	100ng/ml	500ng/ml	900g/ml
Calculated Concentration:	96.7	463.2	909.8
	109.4	497.5	937.0
	105.2	514.2	895.5
	105.0	494.6	889.1
	92.8	490.1	946.7
	93.3	518.4	892.4
Mean:	100.4	496.3	911.7
SD:	7.0	19.7	24.5
CV%:	6.9	4.0	2.7

TABLE 4

Stability of Etoposide in Plasma at -80 °C

Day	Concentration (ng/ml)		
	100.0	500.0	900.0
0	96.9	502.1	903.7
1	95.0	497.2	910.2
7	101.1	519.4	921.6
30	94.4	518.8	893.5
60	106.3	506.8	932.0
120	98.6	479.5	896.3
180	100.7	480.3	879.9

sample collected 48 hours after termination of an etoposide infusion (300mg/m^2/day x 5 days). The retention times for etoposide and teniposide are 6.01 and 11.77 respectively.

The precision of the method over the entire working concentration range was determined with the analysis of spiked quality control samples. Tables 2 and 3 show the intra- and inter-day precision and coefficient of variation (CV) for three concentrations of etoposide. As indicated, the CVs for the intra-day and inter-day studies were below 7%.

Recovery of etoposide from plasma was similar to that reported by other investigators using methylene chloride as the extracting solvent (6,9,10). The mean extraction recovery (N = 3) for 100, 500, and 900ng/ml etoposide spiked plasmas was 94.3, 93.4, and 93.7% respectively. Plasma samples spiked with etoposide and stored for up to 6 months at -80 °C showed no appreciable degradation (table 4).

DISCUSSION

Since this assay has become available, it has been our practice to obtain three to four plasma samples (approximatley 12 hours apart) between 12 and

36 hours after completion of high-dose etoposide therapy. Healthy bone marrow is usually infused at least 60 hours after completion of etoposide which allows one full day to obtain analytical results from patient plasma samples. Linear regression analysis of the etoposide plasma concentration-time points is then used to estimate the concentration at the scheduled time of transplantation. Delay of transplantation for one or more days is considered for those patients with estimated plasma etoposide concentrations exceeding 300 or 400 ng/ml.

Analysis of plasma samples containing etoposide with fluorescence detection is both specific and sensitive. This mode of detection has advantages over both UV and electrochemical detection in that it is in general more sensitive than UV analysis and does not require the care and diligence needed to successfully operate and maintain an electrochemical detector. The sensitivity of fluorescence detection also approaches that observed for electrochemical detection. The method described here also is relatively simple and does not require any sample cleanup prior to or after sample extraction. Because of its sensitivity and ease of operation, this assay would also be useful for pharmacokinetic studies and for the monitoring of patients receiving conventional doses of etoposide.

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