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## Abstract

Several specific assays have been developed for the measurement of etoposide in biological fluids. As large samples are required for high sensitivity, these systems are not appropriate for a pediatric practice. In the present study, however, an improved method for the determination of serum levels of the anticancer drug etoposide was developed, using high-performance liquid chromatography with fixed-wavelength ultraviolet detection. Etoposide was extracted from serum using dichloromethane. The efficiency of extraction from serum was 85.7 +/- 7.7% for etoposide and 81.1 +/- 8.4% for diphenylhydantoin, the internal standard. The serum concentrations of etoposide were measured in 0.2-ml serum samples. The lower limit of detection was 50 ng/ml. Each measurement was completed within 5 min. The linear quantitation range for etoposide was 0.05-50 microg/ml. This assay presents an alternative method for routine measurement of serum levels of etoposide in the pediatric oncology setting.

**KEYWORDS:** etoposide, serum concentration, reversed-phase high-performance liquid chromatography

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*Original Article*

## Determination of Etoposide Serum Concentrations in Small Pediatric Samples by an Improved Method of Reversed-Phase High-Performance Liquid Chromatography

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Several specific assays have been developed for the measurement of etoposide in biological fluids. As large samples are required for high sensitivity, these systems are not appropriate for a pediatric practice. In the present study, however, an improved method for the determination of serum levels of the anticancer drug etoposide was developed, using high-performance liquid chromatography with fixed-wavelength ultraviolet detection. Etoposide was extracted from serum using dichloromethane. The efficiency of extraction from serum was  $85.7 \pm 7.7\%$  for etoposide and  $81.1 \pm 8.4\%$  for diphenylhydantoin, the internal standard. The serum concentrations of etoposide were measured in 0.2-ml serum samples. The lower limit of detection was 50 ng/ml. Each measurement was completed within 5 min. The linear quantitation range for etoposide was 0.05–50  $\mu\text{g/ml}$ . This assay presents an alternative method for routine measurement of serum levels of etoposide in the pediatric oncology setting.

**Key words:** etoposide, serum concentration, reversed-phase high-performance liquid chromatography

**E**toposide, a semi-synthetic derivative of podophylotoxin, is used in treating patients with a variety of malignant tumors [1, 2]. The cytotoxicity of etoposide is dependent serum concentrations of the drug and the duration of exposure [3]. Previous studies suggest that the steady-state concentration or the area under the concentration curve attained as a result of a continuous infusion of etoposide is related to its toxicity [4, 5]. Recent evidence suggests that monitoring etoposide plasma concentrations could help to individual-

ize its dosage, and thus reduce hematologic toxicity while optimizing the dose intensity [6]. Several specific high-performance liquid chromatographic (HPLC) assays have been developed over the past 15 years for the measurement of etoposide in biological fluids [2, 7–11]. As large samples are required for high sensitivity, these systems are not appropriate for a pediatric oncology practice. Our objective was to develop a convenient and sensitive method of HPLC that would be sufficiently reliable for pharmacokinetic studies and for monitoring the serum levels of etoposide during the treatment of malignant disease in children.

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## Materials and Methods

**Chemicals and materials.** Etoposide (Rastel<sup>®</sup>) of pharmaceutical purity was kindly supplied by Nihon Kayaku Inc. (Tokyo, Japan). Distilled water, acetonitrile, dichloromethane, and methanol were of liquid chromatographic grade (Nacalai Tesque, Kyoto, Japan). The internal standard, diphenylhydantoin (DPH, Nacalai), was of analytical reagent grade. Control sera were obtained from healthy volunteers. One-milliliter samples of blood for determinations of serum levels of drug were drawn into polystyrene tubes, then centrifuged at 3000 rpm for 5 min to separate the serum. Sera were stored in polystyrene tubes and frozen at  $-20^{\circ}\text{C}$  until assay.

**Standards and control samples.** Standards and control samples were prepared from a stock solution of  $1000\ \mu\text{g/ml}$  etoposide in a methanol-water (50:50) solution that was added to drug-free pooled serum to give final concentrations of 0, 0.5, 1.0, 5.0, 10.0, 15.0, and  $25.0\ \mu\text{g/ml}$  of serum. Quality-control samples for monitoring within-run and between-run imprecision were similarly prepared but from different stock standards. Standards and controls were aliquoted into 1.5-ml polystyrene tubes and stored at  $-20^{\circ}\text{C}$  until assay.

**Extraction and assay procedures.** Samples for analysis were extracted as follows. A  $20\text{-}\mu\text{l}$  volume of DPH at a concentration of  $100\ \mu\text{g/ml}$  was added to 0.2 ml of serum in a 10-ml glass-stoppered centrifuge tube. After 5 ml of dichloromethane was added, the mixture was shaken for 10 min, then centrifuged at 1500 rpm for 5 min. To another 10-ml clean glass tube was transferred 4.0 ml of the dichloromethane layer, which was evaporated to dryness at ambient temperature in a vacuum evaporator. The residue was reconstituted in  $100\ \mu\text{l}$  mobile phase of the HPLC, and was subjected to HPLC.

**Instrumentation.** Chromatographic determinations of etoposide and DPH were performed using a Chromatopac (Shimadzu, Kyoto, Japan) consisting of the following: an LC-6AD pump coupled to an injector, 9E (Shimadzu), with a  $100\text{-}\mu\text{l}$  fixed loop and an SPD-6A variable-wavelength ultraviolet detector. Data output was monitored with a C-R4A integrator.

**Chromatographic conditions.** The analytical column [ODS-M (octadecylated silica-gel column), Shimadzu], was 150-mm long, with an internal diameter of 6.0 mm and a particle size of  $5\ \mu\text{m}$ . The guard column used, ODS-E (Shimadzu), was 10-mm long with an

internal diameter of 4.0 mm and a particle size of  $5\ \mu\text{m}$ . These were covered with a column heater equipped with a column oven (CTO-6A). The column flow-rate was set at 1.5 ml/min, and the precolumn and column temperatures were maintained at  $40^{\circ}\text{C}$ . The detection wavelength was determined at 229 nm. Analyses were performed in the mobile phase that consisted of methanol, distilled water, and acetonitrile (55:42:3, v/v/v). HPLC was performed on  $20\text{-}\mu\text{l}$  serum samples.

**Calibration curve.** Calibration was performed by the internal standard method. A calibration curve for etoposide was constructed by plotting the relationship between the serum concentrations of etoposide and the etoposide peak area to DPH peak-area ratios. Serum samples were prepared containing 0, 5, 10, 20, and  $40\ \mu\text{g/ml}$  of etoposide mixed with  $100\ \mu\text{g/ml}$  of DPH.

**Extraction efficacy.** The extraction efficacy of etoposide and DPH was calculated by comparing the peak area obtained after injection of the theoretical amount of each compound with that obtained after injection of the extracts obtained from the serum samples.

**Assay validation.** The assay was validated by establishing within-day and between-day variations. To establish within-day variation, 5 samples sets were prepared on the same day. To determine the between-day variation, the assay was repeated monthly for 6 months.

**Statistics.** Results are presented as the mean  $\pm$  S.D. Correlations were calculated by Spearman's rank correlation test. A level of  $P < 0.05$  was accepted as statistically significant.

## Results

**Chromatography.** Chromatograms of a standard sample with  $50\ \text{ng/ml}$  etoposide in solvent and extracted serum sample spiked with etoposide ( $2\ \mu\text{g/ml}$ ) showed no interference from other UV-absorbing substances (Fig. 1). This system typically gave retention times of  $3.4 \pm 0.2$  min for etoposide and  $4.7 \pm 0.1$  min for DPH. The overall chromatographic run could be carried out within 5.0 min.

**Extraction efficiency.** The extraction efficiency of etoposide in serum was  $85.7 \pm 7.7\%$  at a serum concentration of  $5.0\ \mu\text{g/ml}$ . The extraction efficiency of DPH, determined using etoposide as an internal standard, was  $81.1 \pm 8.4\%$  for serum.

**Linearity.** The calibration curve showed a linear correlation between the etoposide/DPH area ratio and

serum concentrations of etoposide (Fig. 2).

**Assay validation.** The method described showed a lower limit of detection for etoposide in serum of 50 ng/ml. The within-day variation was 4.30% in serum at levels of 0.5, 5.0, and 15.0  $\mu\text{g/ml}$  ( $n = 15$ ). The between-day variation was 5.46% in serum at levels

of 0.5, 5.0, and 15.0  $\mu\text{g/ml}$  ( $n = 18$ ).

## Discussion

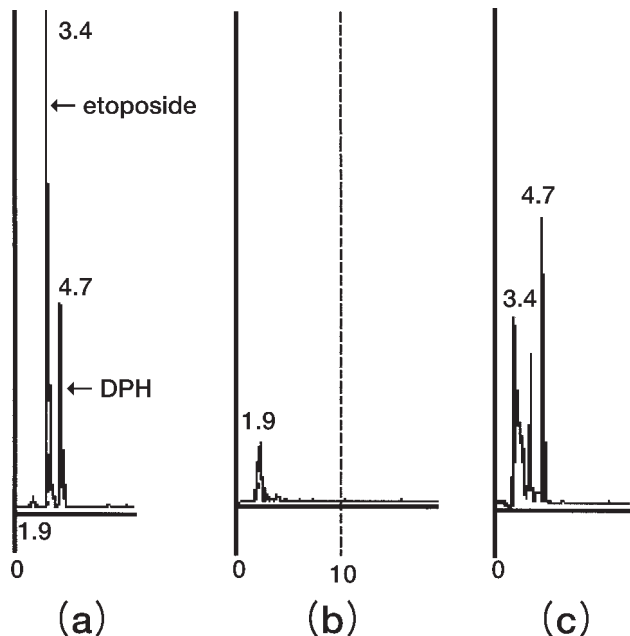
Several HPLC methods [12] for determining serum levels of etoposide have been described since the first report in 1981 by Strife *et al.* [9]. Harvey *et al.* have developed an assay to shorten analysis time [2]. The lower quantitation limit of their assay was 100 ng/ml for 1.0 ml of plasma. The HPLC method reported by Holthuis *et al.* utilized UV detection and had a limit of quantitation of 30 ng/ml [13]. Their assay required a plasma sample of as much as 1.0 ml. Boos *et al.* analyzed the etoposide concentrations by HPLC, which required a serum sample of only 0.19 ml, but the detection limit was 200 ng/ml [14]. Sinkule *et al.* have described an HPLC method having a lower limit of detection of 20 ng/ml utilizing a plasma sample of 0.5 ml [15]. Saita *et al.* have described an enzyme-linked immunosorbent assay (ELISA) for etoposide capable of measuring as little as 40 pg/ml [16]. However, as this method requires a serum sample of as much as 10 ml, it is not useful for pediatric practice. Using the combined method of liquid chromatography and mass spectrometry established by Danigel *et al.*, blood samples of 8–10 ml are required [17].

Although these assays may be useful for monitoring serum etoposide levels in adults, they emphasize sensitivity and simplicity, but not small sample size. They are therefore not appropriate for the detection of serum etoposide concentrations in children.

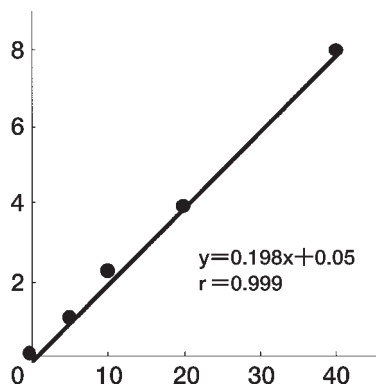
The present HPLC method was established by Fujitaka *et al.* for the quantitation of glucocorticoids [18]. The method can be used to determine the serum concentrations of etoposide in a minimum sample size of 0.2 ml with a detection limit of 50 ng/ml. This small amount of serum can be obtained easily and relatively non invasively from infants and young children with cancer.

The lower limit of detection of 50 ng/ml in the present system was found to be adequate for sample measurement for up to 24 h following intravenous doses of etoposide within the dosage range commonly used clinically in children ( $> 50 \text{ mg/m}^2$  body surface area). This method requires less than 6 min to analyze one sample, which is as simple and accurate as the earlier methods.

In conclusion, in view of the reduced invasiveness and high analytical accuracy of the method described herein, coupled with its simplicity, this assay presents an alterna-



**Fig. 1** Chromatograms of (a) a standard 50 ng/ml etoposide in solvent and extracts obtained from (b) drug-free human serum and (c) a human serum sample with added etoposide (2  $\mu\text{g/ml}$ ) in which x is the time (min) and y is the peak height.



**Fig. 2** Calibration curve for etoposide in serum in which x is the concentration of etoposide ( $\mu\text{g/ml}$ ) and y is the ratio of the peak area of etoposide to that of DPH.

tive method for routine measurement of serum levels of etoposide in a pediatric oncology setting.

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