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Short communication

Assay for etoposide in human serum using solid-phase extraction and high-performance liquid chromatography with fluorescence detection

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Abstract

An HPLC assay for etoposide in human serum was developed. Serum, spiked with podophyllotoxin (internal standard), was treated with sodium dodecyl sulphate prior to solid phase extraction. Analysis was performed on a 300×3.9 mm Bondclone 10 C18 column coupled with a fluorometric detector (λ_{ex} 230 nm, λ_{em} 330 nm). The retention times for etoposide and podophyllotoxin were 14 and 28 min respectively. The range of assay was 0.5 to 20 μg/ml with a detection limit of 0.2 μg/ml. This assay is suitable for use in clinical studies with etoposide. © 1998 Elsevier Science B.V.

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1. Introduction.

Etoposide, a semisynthetic derivative of epi-podophyllotoxin, has broad spectrum antitumour activity and is principally used in the treatment of leukemias, lymphomas and lung cancer. The drug is highly (90–95%) bound to plasma proteins, metabolized by P450-mediated hepatic metabolism and excreted in both bile and urine [1].

The pharmacokinetics of etoposide has been extensively investigated in cancer patients. Such studies typically employ high-performance liquid chromatography (HPLC) with UV, fluorescence or electrochemical detection; the latter two methods providing greater sensitivity than UV detection.

These HPLC methods require sample preparation which ensures release of etoposide from plasma protein binding sites and separation from the biological matrix. This has variously been accomplished using liquid–liquid extraction with chloroform [2,3], dichloromethane [4], and ethylene dichloride [4]. Alternatively, solid phase extraction has been described using a reversed-phase stationary phase, and simple purging with water followed by methanol to

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elute etoposide [5,6]. Finally, a procedure permitting direct on-line clean-up of plasma has been reported [7]. This technique included the addition of sodium dodecyl sulphate (SDS) to solubilize plasma proteins and to release protein-bound etoposide.

The present assay combines the use of solid phase extraction [5,6] with SDS addition [7] to achieve greater efficiency in sample preparation. In addition, to date, teniposide has commonly been used as an internal standard for etoposide HPLC analysis, particularly with fluorescent detection. In its pure form, this compound is relatively inaccessible, obtained only from the manufacturer. In the present method, teniposide is replaced by a widely available compound, podophyllotoxin.

2. Experimental

2.1. Chemicals and reagents

Etoposide, podophyllotoxin, 1-heptansulphonic acid, anhydrous monobasic potassium phosphate and ammonium acetate were purchased from Sigma Chemical (St Louis, MO, USA). SDS was a product of Bio–Rad Laboratories (Richmond, CA, USA). Blank human serum was acquired from ICN Pharmaceuticals (Costa Mesa, CA, USA). Methanol, HPLC grade, was purchased from EM Science (Gibbstown, NJ, USA).

2.2. Preparation of standards

Standard solutions of etoposide and podophyllotoxin, as internal standard (IS), were prepared in methanol. Calibration curves for etoposide in human serum were made by adding standard solutions to blank human serum yielding concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 15 and 20 μg/ml.

2.3. Extraction procedure.

Serum samples (500 μl), 500 μl of 20 m*M* ammonium acetate (pH 5.5), 50 μl of SDS (760 m*M*) and 50 μl of IS (0.8 μg/ml) were added to polypropylene tubes. The contents of the tubes were mixed and applied to preconditioned 3 ml/500 mg Bond–Elute PH cartridges (Varian, Harbor City, CA, USA). Preparation of the cartridges for extraction was accomplished by rinsing with 6 ml of methanol followed by 3 ml of 20 m*M* ammonium acetate (pH 5.5). After loading with the serum sample, serum matrix components were eluted with 3 ml of 20 m*M* ammonium acetate and 3 ml of 10% methanol in water. Etoposide and IS were then eluted with 2 ml of methanol and evaporated to dryness under vacuum at 43°C in an SC-100A SpeedVac concentrator (Savant Instruments, Holbrook, NY, USA). The residue was reconstituted in 150 μl of 36% methanol in water.

2.4. Chromatography

The HPLC system used for analysis of human serum samples included an LC-10-T solvent delivery system, an SCL-10A system controller, an SIL-10AXL auto injector, an RF-535 fluorometer and an SR501 chromatopac integrator, all from Shimadzu Scientific Instruments (Columbia, MD, USA). Separation of etoposide and IS in serum was performed using a 300×3.9 mm Bondclone 10 C_{18} column (Phenomenex, Torrance, CA, USA). The mobile phase was comprised of methanol, 40 m*M* KH_2PO_4 (pH 6.9) and 0.14 m*M* 1-heptansulphonic acid in the ratio 400:600:6. The flow-rate was 2 ml/min and detection was provided by a fluorometric detector with λ_{ex} 230 nm and λ_{em} 330 nm.

2.5. Quantitation

Concentrations of etoposide in unknown samples were ascertained from the slopes of standard curves of the peak-area ratio (etoposide/IS) versus standard etoposide concentration. Standard curve slopes were determined by least-squares regression analysis (Corel Quattro Pro 6.0, Corel Corporation, Ottawa, Ontario, Canada).

2.6. Assay specifications

The extraction recovery of etoposide was determined by comparing the peak area for five extracted human serum samples with that of five unextracted samples of the same amount of the drug prepared in mobile phase. Three concentration levels, 1, 5 and 10

μg/ml, were investigated. The percentage extraction recovery was calculated from 100*peak $area_{extracted}$/peak $area_{unextracted}$.

The intra-day precision of the assay in serum was estimated by assaying five samples at each concentration level, 1, 5 and 10 μg/ml. For the inter-day assay precision, samples with concentrations (ranging from 0.5 to 20 μg/ml) were analyzed on five separate days. Precision is defined as relative standard deviation (R.S.D.). Accuracy was calculated by comparing measured etoposide concentrations to the known values.

3. Results and discussion

Chromatograms of blank human serum (A), human serum with etoposide and IS (B), blank patient plasma (C), and plasma of patient treated with etoposide and spiked with IS (D) are depicted in Fig. 1. Neither the commercially obtained serum blank nor blank patient plasma demonstrated interfering peaks (Fig. 1A,C). However plasma from patients receiving etoposide therapy manifested a potentially interfering peak. In order to achieve baseline separation from this interfering peak, the

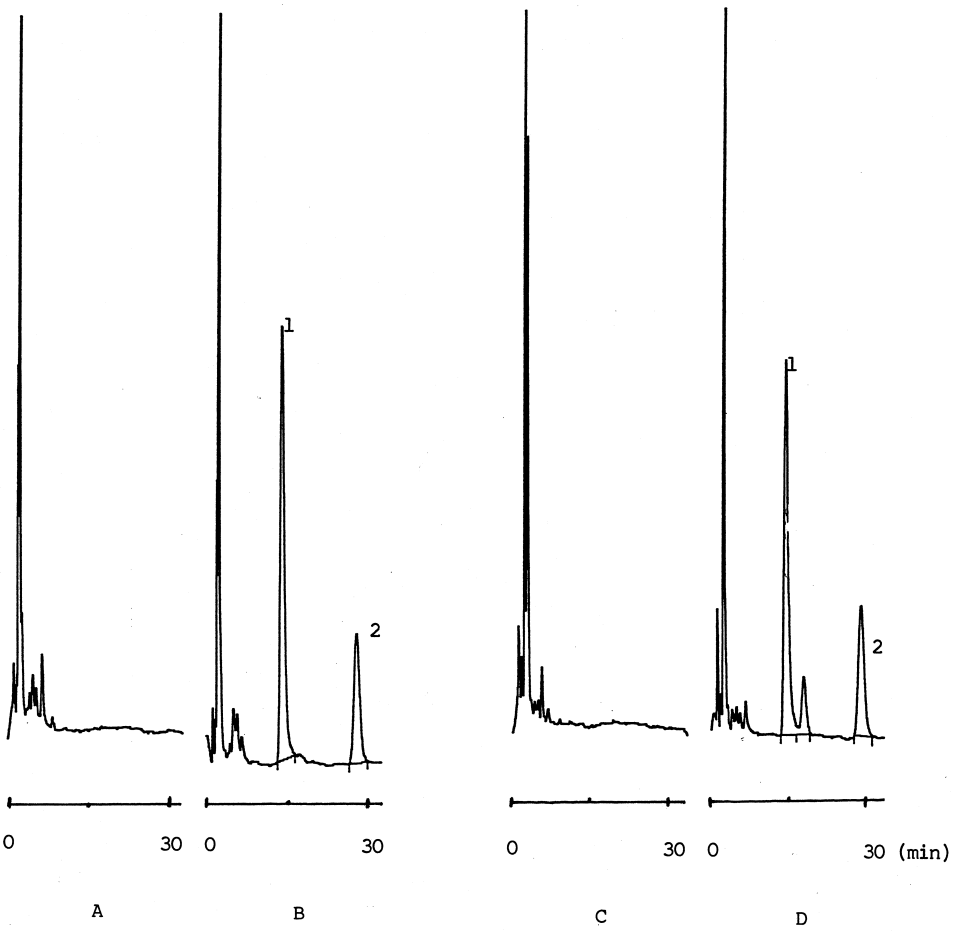

Fig. 1. Chromatograms obtained by solid-phase extraction and fluorometric detection: (A) blank serum, (B) same serum spiked with etoposide (1) in concentration 5 μg/ml and podophyllotoxin (2) as internal standard, (C) plasma from patient before and (D) after treatment with etoposide.

methanol content of the mobile phase was established at 40%. This yielded the current retention times for etoposide and IS in clinical samples of 14 and 28 min respectively (chromatogram D, Fig. 1). The average value of the area of the potentially interfering peak ranged from four to 16% of the etoposide peak area. The identity of the substance associated with the peak has yet to be elucidated.

Mean recoveries from serum samples containing 1, 5 and 10 μg/ml of etoposide and IS were 90, 88, 89 and 86% respectively.

Calibration curves for etoposide were calculated by linear least-squares regression in the range 0.5–20.0 μg/ml [$Y=0.51X-0.017$, SE=0.003, $r^2=0.999$; X denotes the independent variable, i.e. the concentration of etoposide in serum (μg/ml); Y denotes the dependent variable, i.e. ratio of the peak-area values; SE and r^2 denote the estimated standard error and the correlation coefficient].

The accuracy, and the inter-day and intra-day precision of the method are presented in Table 1. These values are satisfactory for the determination of etoposide pharmacokinetics in clinical settings. The extraction efficiency of the method is comparable with liquid–liquid extraction [3,8–10] and solid phase extraction on Bond Elute CN cartridges [6]. To achieve this extraction efficiency it was found that initial pretreatment of serum is necessary: specifically mild acidic conditions and the use of SDS in order to release etoposide from the drug–protein complex. These conditions also permitted reuse of cartridges for several extractions including those experiments used to assess inter-day precision.

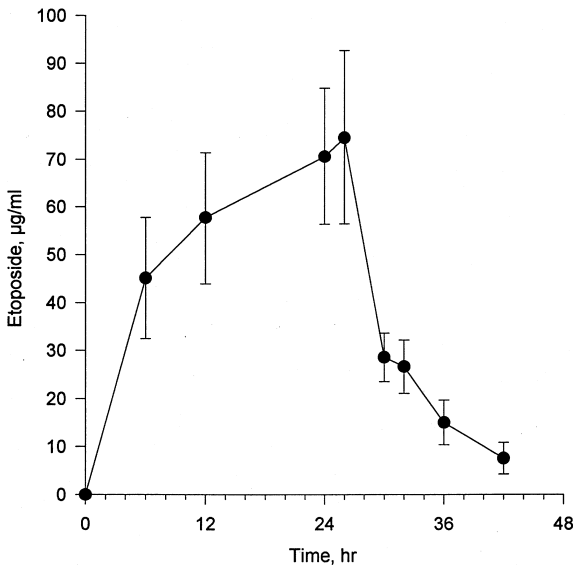

Fig. 2. Plot of mean etoposide serum concentrations (±SD) versus time during and after administration of 1500 mg/m^2 etoposide as a 24 h i.v. infusion.

The detection limit reported, 0.5 μg/ml, is comparable to that obtained with other methods using fluorescence detection [2,9–11].

Serum samples were collected from five paediatric patients undergoing bone marrow transplantation. Blood samples were drawn during and after a 24 h

Table 1
Accuracy and precision of assay of etoposide in human serum

Nominal concentrations (μg/ml)	Measured concentrations[a] (μg/ml)	Accuracy	Precision[b]	
		Inter-day (%)	Inter-day (%)	Intra-day (%)
0.5	0.51±0.06	102	12.9	
1	1.00±0.08	100	7.8	3.1
2	1.99±0.08	99.5	4.1	
5	5.00±0.41	100	8.3	2.1
10	10.07±0.18	101	1.8	5.1
15	14.91±0.49	99.0	3.3	
20	19.95±0.93	100	4.7	

[a]mean±SD.
[b]$n=5$.

infusion. The plasma concentration–time profile of etoposide in these patients is shown in Fig. 2. Plasma samples with drug concentrations above the etoposide calibration curve were diluted prior to final estimation.

In summary, an HPLC assay for etoposide was developed which included solid-phase extraction and a more readily available internal standard. The proposed sample preparation provides clean chromatograms contributing to high analysis precision. The assay can be readily used for monitoring this important antineoplastic agent in the setting of conventional and high dose therapy.

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