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Short Communication

Determination of etoposide in blood by liquid chromatography with electrochemical detection

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ABSTRACT

A liquid chromatographic (LC) method for determination of etoposide in dog blood is described. The technique includes solvent extraction of etoposide using a dichloroethane–hexane mixture and reconstitution of the drug in an aqueous reconstitution solution. The samples are analyzed by reversed-phase LC with electrochemical detection. Validation of the method demonstrated good sensitivity, precision and reproducibility. The method is useful for the study of etoposide pharmacokinetics in the dog.

INTRODUCTION

Etoposide is a semisynthetic epipodophyllotoxin derivative, active against a variety of malignancies [1]. It is the most active single agent for small-cell lung cancer [2], and also approved by the Food and Drug Administration for treating testicular carcinoma. The currently available dosage form is non-aqueous parenteral solution. The oral dosage form is highly desired for long-term out-patient administration, but has not yet been satisfactorily formulated. Several investigational oral formulations including hydrophilic, soft gelatin capsule containing etoposide solution, lipophilic capsule of etoposide suspension, and drinking ampules, have been evaluated [3–5]. However, all were found to have the drawback of low bioavailability. In order to improve the oral bioavailability using formulation approaches, a novel, nanocrystalline formulation was developed in our laboratory. To conduct preformulation studies with the nanoparticulate formulation, it was necessary to develop a high-performance liquid chromatographic (LC) method for detection of low levels of etoposide in whole blood.

Several LC methods have been employed for determination of etoposide in plasma [6–15]. These methods included ultraviolet absorption and spectrofluorimetry which provide detection limits of etoposide from 0.03 to 0.5 μg/ml in plasma. Electrochemical detection significantly decreases these limits [16, 17]. None of the methods was applicable to determination of etoposide in whole blood. Therefore, we have developed a simple, rapid assay for etoposide with electrochemical detection using teniposide [4′-dimethylepipodophyllotoxin 9-(4,6-O-thenylidene-β-D-glucopyranoside), II] as internal standard in dog

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blood. The method is precise, accurate and sufficiently sensitive to measure low blood levels of the drug encountered in dog blood following oral administration of nanocrystalline etoposide.

EXPERIMENTAL

Chemicals and solutions

Etoposide was purchased from Sigma (St. Louis, MO, USA). Teniposide was kindly provided by Dr. M. Suffness (National Cancer Institute, Bethesda MD, USA). Other chemicals of HPLC grade were obtained from Fluka (Ronkonkoma, NY, USA).

Etoposide stock solution was prepared by dissolving 2 mg of etoposide in 4 ml of methanol to obtain a 0.5 mg/ml solution. To obtain working standards, 40 μl of the stock solution were added to 3960 μl of methanol and then serially diluted to obtain 12 standards ranging from 50 μg/ml to 24 ng/ml. The blood standards were prepared by spiking 20 μl of the corresponding working standards into 180 μl of whole dog blood to obtain concentrations in the range from 5000 ng/ml to 2.4 ng/ml.

Quality control samples, used to validate the assay, were prepared by spiking the corresponding working standards into the blood to obtain concentrations of 1000, 200, and 50 ng/ml.

Stock solution of 0.5 mg/ml teniposide (internal standard) was prepared in a way similar to the preparation of etoposide solutions. The working internal standard/extraction solvent was prepared by adding 30 μl of 0.5 mg/ml stock internal standard to 100 ml of a dichloroethane–hexane mixture (1:1, v/v) to obtain a 150 ng/ml solution.

Assay

The samples were processed by placing 200-ml aliquots of the blood containing etoposide as a standard or unknown into 1.5 ml polypropylene centrifuge tubes. To these tubes, 1 ml of the dichloroethane–hexane mixture with the internal standard solution was added and the resulting mixture was vortex-mixed for 1 min, followed by centrifugation for 3 min (Microfuge, 15 000 *g*). The supernatant was transferred into a polypropylene centrifuge tube and evaporated in a Savant Speed-Vac evaporator for 30 min at 40°C. After evaporation, the samples were reconstituted with 60 ml of the reconstitution solution. The solution was prepared by mixing 40 ml of acetonitrile with 60 ml of water and 0.4 ml of 0.65 *M* sodium citrate, pH 4.0. The reconstituted samples were then transferred into WISP autosampler inserts and injected into the LC system (30 μl injection).

Chromatography

The apparatus consisted of a Waters Model 510 pump, refrigerated 712 WISP autosampler, Microbondapak Phenyl guard (10 mm × 3.9 mm I.D., 10 μm) and analytical columns (150 mm × 3.9 mm I.D., 10 μm), and an ESA 5100A electrochemical detector (ESA, Bedford, MA, USA) equipped with a Model 5020 guard cell and Model 5011 dual electrode analytical cell. The guard cell, set at +0.7 V, was installed between the pump and autosampler. The up-stream electrode (screening electrode) of the analytical cell was set at +0.2 V and the downstream electrode was set at +0.45 V *versus* Ag/AgCl. Mobile phase A was prepared by mixing 77 ml of 0.65 *M* sodium citrate, 573 ml of water, and 350 ml of acetonitrile. Mobile phase B was prepared by mixing 77 ml of 0.65 *M* sodium citrate, 273 ml of water, and 700 ml of acetonitrile. Etoposide and teniposide were eluted under isocratic conditions (mobile phase A for 7 min), followed by a 4 min wash (mobile phase B) and re-equilibration with mobile phase A for 9 min. The flow-rate was 2 ml/min with the LC column at ambient temperature.

RESULTS

Chromatography

Representative chromatograms of etoposide in rat blood extracts are shown in Fig. 1. The drug-free blood sample spiked with teniposide is displayed in Fig. 1A. A typical chromatogram of a blood sample obtained from a dog 120 min after oral administration of 5 mg/kg etoposide and spiked with teniposide is shown in Fig. 1B. Total

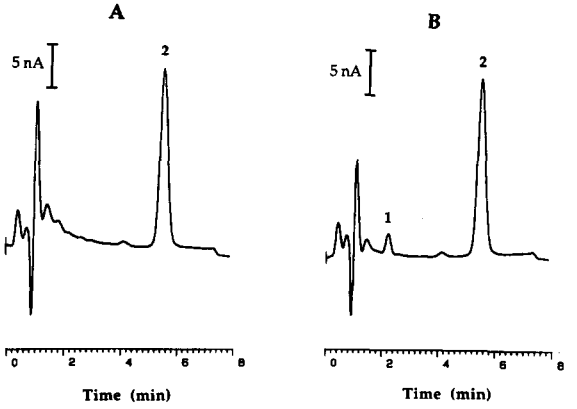

Fig. 1. Representative chromatograms of dog blood extracts spiked with 3 μg/ml internal standard, teniposide. (A) Drug free sample of dog blood; (B) blood sample taken 10 h after oral administration of 2 mg/kg etoposide to the dog as the nanoparticulate formulation. Amount of etoposide is 3 ng on column. Conditions are as described in the Experimental section. Peak identification: (1) etoposide, (2) teniposide (internal standard).

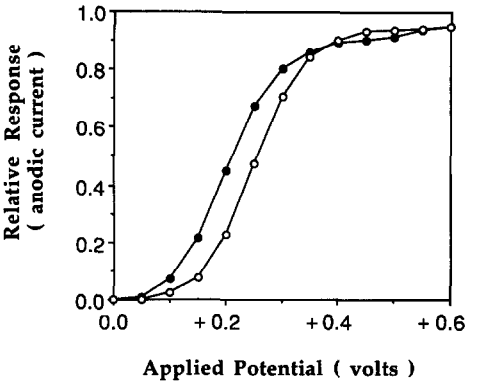

Fig. 2. Current–potential profile (hydrodynamic conditions) for etoposide and teniposide (32 ng on column). (●) Etoposide, (○) teniposide. Conditions are as described in the Experimental section.

chromatography time is 20 min, the peaks are symmetrical and well resolved, and the baseline is clean and free from interfering peaks.

A hydrodynamic voltammogram of etoposide and teniposide (Fig. 2) shows that a peak-height plateau for etoposide is reached at +0.4 V. This voltage was chosen as the detection potential for this assay. The potential of +0.2 V at which the current reaches about 10% of its plateau value was chosen as the screen potential.

Validation of assay

The method was validated by performing replicate analyses (n = 5) of dog blood pools spiked with etoposide (50, 200 and 1000 ng/ml) on three separate days. Concentrations were determined by comparison with a standard curve prepared on the day of analysis. The precision as well as the overall accuracy of the method were estimated (Table I). The within-day precision for both compounds, defined as the mean of the daily coefficients of variation at each concentration

TABLE I

ACCURACY AND PRECISION DATA FOR THE DETERMINATION OF ETOPOSIDE IN THE DOG BLOOD

Concentration (μg/ml)	Day	Assay concentration (mean ± S.D., n = 5) (μg/ml)	Precision[a] (%)		Accuracy[a] (%)
			Within-day (n = 5)	Between-day (n = 15)	
0.050	1	0.049 ± 0.004	7.79	7.54	102.6
	2	0.052 ± 0.003			
	3	0.053 ± 0.005			
0.200	1	0.196 ± 0.010	4.06	4.04	102.3
	2	0.210 ± 0.007			
	3	0.208 ± 0.008			
1.000	1	0.969 ± 0.005	1.54	2.67	97.2
	2	0.981 ± 0.006			
	3	0.967 ± 0.034			

[a] The within-day, between-day precision and accuracy are defined in the text.

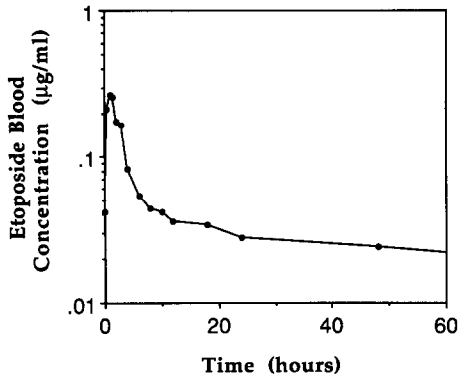

Fig. 3. Blood etoposide levels following oral administration of 2 mg/kg etoposide in dog.

(n = 5), ranged from 1.5 to 7.8%. The between-day precision, expressed as the coefficient of variation of the pooled three-day data at each concentration (n = 15), observed for both compounds was in the range of 2.7 to 7.5%. The accuracy of the method expressed as the ratio of predicted to actual concentration (C-ratio) was in the range of 97.2% to 102.6%.

Etoposide was stable in the reconstitution solvent over 48 h at +10°C and for over 3 months in the blood at −70°C.

Calibration linearity, limit of detection and limit of quantitation

Peak-height ratios of drug to internal standard were plotted *versus* drug concentrations. The slope and intercept of the standard curve were estimated by least-squares linear regression. Standard curves from 2.4 to 5000 ng/ml etoposide were linear and gave correlation coefficients better than 0.997. The method detection limit (MDL) for etoposide, determined at a signal-to-noise ratio of 3, was 2.4 ng/ml. The method quantitation limit, defined as 3 × MDL, was 7.5 ng/ml etoposide. The detection limit on column was 0.24 ng of etoposide.

Reproducibility and recovery

To determine the recovery, two dog blood pools were prepared at concentrations of etoposide of 200 and 1000 ng/ml each, replicate samples (n = 5) were processed as described in the Experimental section and compared to concentrations of the aqueous unextracted standards. The total recovery of etoposide ranged from 78% to 94%.

DISCUSSION

The assay was developed to determine etoposide in whole blood in order to carry out pharmacokinetic studies. Most of the previously reported methods have been developed for extraction of etoposide from the plasma [6–16]. However whole blood presents a different set of challenges. The high viscosity and degree of clotting of the blood samples makes the use of solid phase extraction [16] impractical. The solvents used for liquid extraction and reported to give the highest recovery were chlorinated hydrocarbons — dichloroethane and chloroform. Both of these solvents are heavier then blood and, after extraction, form a clear bottom layer which is difficult to aspirate without contamination with the upper blood layer. We found that even a small amount of red blood cells in the extraction solvent caused large contamination peaks. Using a dichloroethane–hexane mixture (1:1, v/v) helps to circumvent the problem of contamination. The produced solvent had a specific gravity of 0.965 g/ml and its extraction properties were equal to those of dichloroethane. Following separation, the solvent forms the upper layer and can be easily aspirated with no risk of contamination.

A representative blood concentration *vs.* time profile following oral administration of 2 mg/kg etoposide in dog is shown in Fig. 3. A wash cycle in each LC run was essential in order to eliminate late eluting peaks. It also helped to maintain the porous graphite electrodes of the Model 5011 analytical cell. The proper mobile phase, wash cycle, and low applied potential allowed the same cell to be used continuously for over four months with no decline in performance. The detector response was reproducible over the four months involved in assay development and sample analysis. The background current did not show any significant increase during the course of an analytical run.

The assay appears to be suitable for the rapid and sensitive determination of etoposide in dog blood, thus allowing accurate estimation of its pharmacokinetic parameters following administration of etoposide to the dog. The technical simplicity, speed and robustness of the method garantee a reliable procedure, which supports the unattended analysis of up to 96 samples per day.

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