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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ETOPOSIDE IN PLASMA USING ELECTROCHEMICAL DETECTION

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SUMMARY

A quantitative analytical method has been established for the determination of a semi-synthetic epipodophyllotoxin, etoposide, in plasma. The method employs reversed-phase high-performance liquid chromatography and electrochemical detection. Sample preparation consisted of extraction with 1,2-dichloroethane followed by phase separation, evaporation of the organic phase, and reconstitution of the residue. Observed recoveries were 76.8 and 87.5% for 50 and 500 ng/ml, respectively. The method had a linear range of 10—1000 ng/ml. Correlation coefficients of 0.997 or greater were obtained during validation experiments and study sample analysis.

INTRODUCTION

Etoposide (VP-16), 4′-demethyl-epipodophyllotoxin-β-D-ethylidene glucoside (Fig. 1), is a semi-synthetic derivative of podophyllotoxin with anti-neoplastic properties. It was introduced into clinical trials in 1973. It has been shown to have activity in childhood leukemias, lymphomas, neuroblastomas, and brain and germ cell tumors, as well as adult lung, brain, bladder and testicular cancers and adult leukemias and lymphomas [1—5].

Scalzo et al. [6] described a method for the determination of etoposide in human plasma by high-performance liquid chromatography (HPLC) with UV detection. The limit of quantitation of that assay was reported at 100 ng/ml, using 2.0 ml of plasma. An HPLC method described by Holthius and Van Oort [7] utilized UV detection and had a limit of quantitation of 30 ng/ml. Etoposide was also analyzed in plasma by reversed-phase HPLC and electro-

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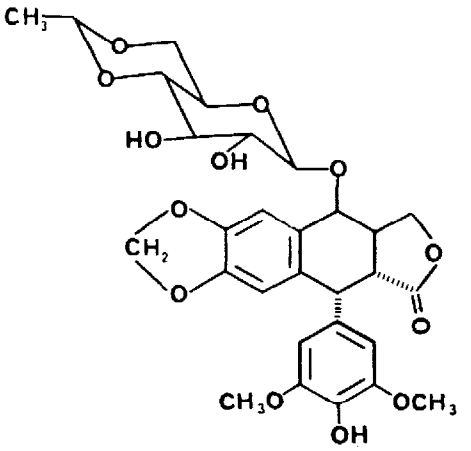

Fig. 1. Structure of etoposide (4'-demethyl-epipodophyllotoxin-β-D-ethylidene glucoside, ethylidene lignan P, VP-16, NSC-141540).

chemical detection [8]. The procedure described in the present paper is a sensitive HPLC assay for etoposide, using reversed-phase separation and electrochemical detection. An application of this procedure is shown by the assay of dog plasma samples following oral administration of etoposide.

EXPERIMENTAL

Apparatus

Chromatography was performed using an IBM (Danbury, CT, U.S.A.) LC/9533 ternary gradient pump. Detection was performed with an Environmental Sciences Assoc. (Bedford, MA, U.S.A.) electrochemical detector equipped with a 5020 guard cell (+0.7 V) and a 5010 standard analytical cell (detector 1: +0.2 V to screen, detector 2: +0.5 V to detect). The autosampler (WISP 710B) and HPLC column (μBondapak CN; 10 μm, cyanopropyl-bonded silica) used were both from Waters Assoc. (Milford, MA, U.S.A.). The recorder used was a Linear 585 from Linear Instruments (Irvine, CA, U.S.A.).

The detector output was digitized by a Hewlett-Packard (Palo Alto, CA, U.S.A.) 18625A analog-to-digital converter and the data were collected and processed by a Hewlett-Packard HP 3357 laboratory automation system computer.

Chemicals and reagents

Etoposide was obtained from the References Standard Laboratory of Bristol-Myers. Acetonitrile and methanol were HPLC grade from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Phosphoric acid (85%), glacial acetic acid and 1,2-dichloroethane were Certified A.C.S. reagents and sodium phosphate, dibasic, and sodium acetate were Certified A.C.S. chemicals, all obtained from Fisher Scientific. Distilled water was deionized and filtered through a Millipore (Bedford, MA, U.S.A.) Milli-Q system. Plasma samples and control dog plasma were obtained from the beagle colony of the Department of Drug Metabolism and Pharmacokinetics (Bristol-Myers, Syracuse, NY, U.S.A.).

The HPLC mobile phase was prepared by dissolving 6.94 g of sodium acetate in 2550 ml of deionized water. The pH of this solution was then adjusted to 4.0 with glacial acetic acid. The solution was thoroughly mixed with 550 ml of acetonitrile. The resulting mixture was filtered through a 0.22-μm Durapore filter.

Acquisition of study samples

The study samples that were analyzed were obtained in the course of performing a bioequivalence study in male beagle dogs (9—12 kg), comparing several different etoposide formulations. A total of 504 samples were collected during the study.

Standard and quality control preparation

Etoposide stock solutions were prepared at concentrations of 100, 10.0 and 1.0 μg/ml in methanol. These solutions were used to spike control dog plasma to prepare standards for the assay. The dog plasma standards were prepared immediately prior to each analytical run.

Quality control samples were prepared prior to the beginning of the study. They were used to validate the assay and to monitor the performance of the assay during the study. For assay validation dog plasma was spiked at etoposide concentrations of 25.3, 316 and 1264 ng/ml. For study sample stability dog plasma was spiked at etoposide concentrations of 29.0, 463 and 1158 ng/ml.

Sample preparation

Standards, quality controls, and samples were processed in batch. The samples were processed by adding 0.50 ml of 0.2 *M* phosphate buffer, pH 8.0 to 1.0 ml of dog plasma. After mixing, 5.0 ml of 1,2-dichloroethane were added and mixed by rotation for 10 min. The layers were then separated by centrifugation at 800 *g* and 4°C for 10 min. The upper aqueous phase was aspirated off and discarded. A 3.50-ml volume of the lower organic phase was transferred to clean tubes and evaporated to dryness with nitrogen. The extraction residues were reconstituted in 200 μl of acetonitrile—0.02 *M* sodium acetate, pH 4.0 with glacial acetic acid (7:3). The resulting solutions were transferred to autosampler vials containing limited volume inserts. The vials were randomised in autosampler trays and 30-μl volumes were injected into the HPLC system for analysis.

Chromatography

The HPLC mobile phase, acetonitrile- 0.02 *M* sodium acetate, pH 4.0 (17.7:82.3), flowed through a μBondapak CN column at a rate of 1.8 ml/min. Detection was performed using dual-series porous graphite electrodes with the first electrode at +0.2 V to screen out interfering peaks, and the second electrode at +0.5 V to detect etoposide. A porous graphite electrode guard cell, set at +0.7 V, was in-line, prior to sample introduction to oxidize the mobile phase components. The retention time of etoposide was between 8 and 8.5 min. The detector gain was 10 × 10 with a response time of 10 s. A background current of less than 0.5 μA was obtained 3—4 h after start-up in detector 2.

Data processing

Detector output was recorded on a strip-chart recorder and by Hewlett-Packard HP 3357 laboratory automation system computer. Each chromatogram was interpreted using the Model 3357 computer software. At the end of each series of analyses, the regression of etoposide peak height versus etoposide concentration in the plasma standard was calculated by least-squares analysis. The concentration of etoposide in the unknown samples was estimated by inverse prediction.

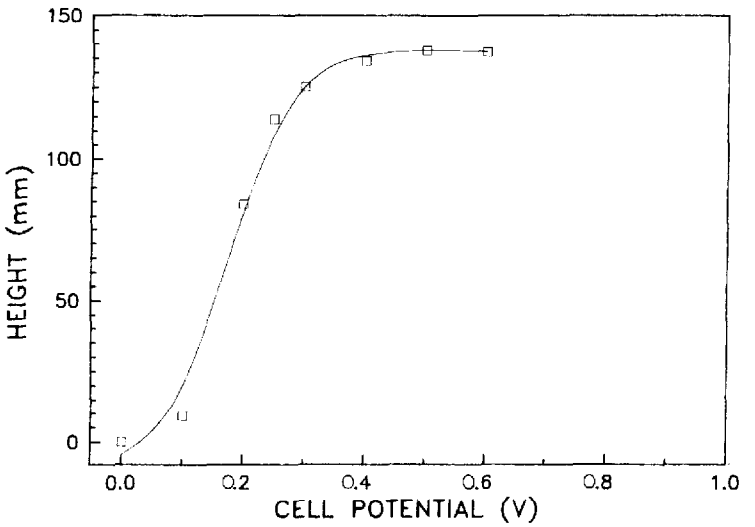

Fig. 2. Hydrodynamic voltammogram for etoposide.

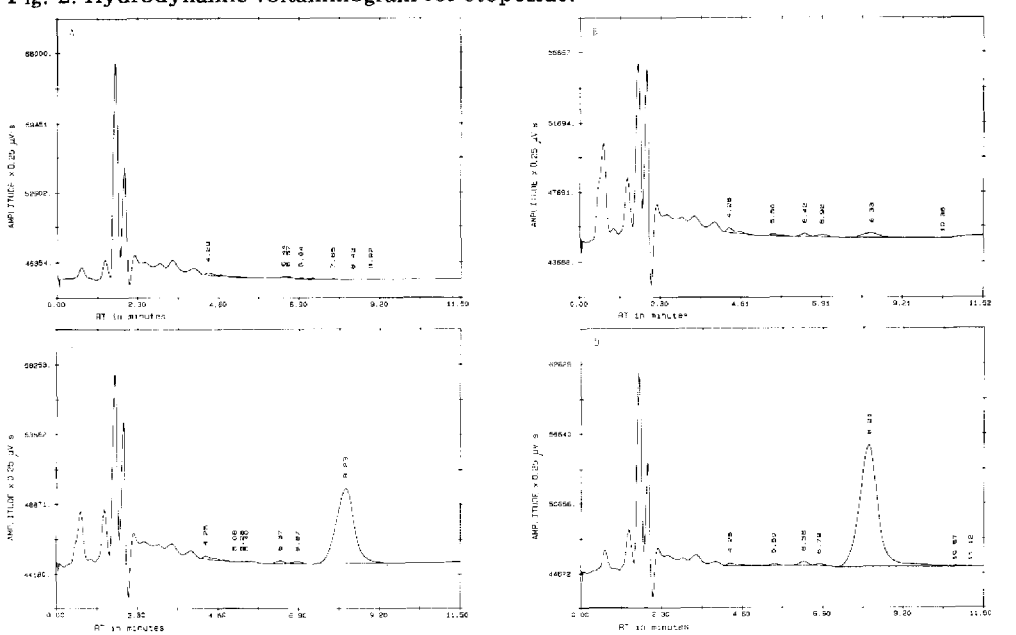

Fig. 3. Chromatograms of extracted dog plasma samples. The etoposide retention time is 8.2—8.3 min. (A) Plasma blank; (B) standard 10 ng/ml etoposide; (C) standard 200 ng/ml etoposide; (D) subject 11, 1 h, TRT No. 2.

TABLE I

LIMIT OF QUANTITATION FOR ETOPOSIDE PLASMA ASSAY

Sample No.	Peak height (μV-s)	
	Blank	Spiked
1	0	296.6
2	30.0	197.2
3	0	218.6
4	0	221.0
5	0	248.2
6	0	216.4
7	0	213.8
8	0	231.5
9	58.8	238.0
10	0	184.9

Statistical data
Mean treatment difference (A — B) = 220.754
Standard error = 12 T = 18 Degrees of freedom = 9
Integral — infinity to absolute T = 1 for T = 18.2225 with 9
Probability of observed difference being real: 1-tail = 1 2-tail = 1

TABLE II

REPRESENTATIVE STANDARD CURVE DATA FOR ETOPOSIDE

Concentration (ng/ml)	Height (μV-s)	Mean height (μV-s)	R.S.D. (%)	Weight	Concentration (ng/ml)	Mean concentration (ng/ml)	Deviation (%)
10.0	233.64	230.80	1.7	0.100	10.4	10.3	3.0
10.0	227.97			0.100	10.2		
20.0	522.44	505.18	4.8	0.050	21.1	20.5	2.5
20.0	487.91			0.050	19.8		
50.0	1304.45	1291.47	1.4	0.020	50.2	49.8	- 0.4
50.0	1278.50			0.020	49.3		
100	2584.57	2585.59	0.1	0.010	97.9	97.9	—2.1
100	2586.61			0.010	97.9		
150	3928.40	3775.10	2.8	0.007	147.9	145.1	—3.3
150	3775.10			0.007	142.2		
200	5375.88	5261.60	3.1	0.005	210.7	197.5	—1.2
200	5147.33			0.005	193.2		
300	8295.89	8001.46	5.2	0.003	310.4	299.5	- 0.2
300	7707.04			0.003	288.5		
500	13784.20	13771.75	0.1	0.002	514.6	514.2	2.8
500	13759.30			0.002	513.7		
1000	27021.00	26711.10	1.6	0.001	1007.1	995.6	—0.4
1000	26401.20			0.001	984.1		

Curve parameter	95% Confidence limit	
	Lower	Upper
Slope = 26.87408	26.52019	27.22796
Intercept = —45.0975	- 83.4862	—6.70874
Correlation coefficient = 1.000		

RESULTS

Validation of assay

A hydrodynamic voltammagram of etoposide (Fig. 2) shows that a peak height plateau is reached at 0.5 V. This voltage was chosen as the detection potential for this assay. The response of the detector to two different levels of etoposide and a blank is illustrated in Fig. 3. Based on the clear separation of response in paired, blank versus spiked samples, a plasma concentration of 10.0 ng/ml etoposide was chosen as the lower limit of quantitation (Table I). Standard curves from this low level to 1000 ng/ml etoposide were linear (see Table II); and gave correlation coefficients better than 0.997. The intra-assay precision, as measured by relative standard deviation (R.S.D.), was 11.7, 2.7 and 3.7% for the theoretical concentrations 25.3, 316 and 1264 ng/ml. The predicted concentrations of these samples were accurate to within 8.8, 5.5 and 4.3%, respectively (see Table III). Table IV shows that etoposide was stable in the dissolution solvent over 48 h. Quality control samples were made to test the long term storage stability of the samples. Table V shows that etoposide was stable in plasma samples stored for over one month at −30°C. The assay was checked for interferences from both etoposide precursors and potential degradation products (Table VI). A total of 26 control dog plasma samples from eight different dogs were also checked for interfering substances. In both cases no interference was observed. Pharmacokinetic data, as determined by this assay, for dogs after oral administration of etoposide are shown in Table

TABLE III

INTRA-ASSAY ACCURACY AND PRECISION FOR THE ETOPOSIDE DOG PLASMA ASSAY (n = 9)

Theoretical concentration (ng/ml)	Mean observed concentration (ng/ml)	R.S.D. (%)	Deviation (%)
25.3	27.5	11.7	8.8
316	333.5	2.7	5.5
1264	1319.0	3.7	4.3

TABLE IV

STABILITY OF THE RECONSTITUTED PLASMA EXTRACTION RESIDUES (n = 6) FOR THE ETOPOSIDE DOG PLASMA ASSAY

Time (h)	Mean peak heights (μV-s)	Standard deviation	R.S.D. (%)
0	5699.03	196.81	3.5
6	5356.96	96.82	1.8
12	5271.17	105.89	2.0
18	5209.14	41.75	0.8
24	5264.57	108.53	2.1
36	5092.56	202.50	4.0
48	5167.51	155.04	3.0

TABLE V

INTER-ASSAY PRECISION AND ACCURACY FOR THE ETOPOSIDE DOG PLASMA ASSAY

Assay day	Replicate No.	Nominal concentration (ng/ml)		
		29.0	463.3	1158.3
Individual quality controls				
1	1	29.6	507.3	1230.5
	2	23.4	528.0	1246.0
2	1	29.9	504.6	1330.0
	2	29.1	486.6	1249.5
5	1	29.9	483.7	1265.5
	2	36.1	522.5	1260.5
7	1	30.2	502.6	1299.5
	2	24.4	493.5	1187.0
9	1	30.9	495.5	1248.5
	2	25.3	513.7	1221.5
12	1	30.9	506.9	1202.5
	2	32.2	488.4	1244.0
14	1	30.9	517.9	1230.5
	2	30.8	518.8	1272.5
16	1	33.0	525.3	1263.0
	2	30.5	517.2	1215.5
20	1	26.6	511.2	1204.5
	2	29.6	496.0	1259.0
22	1	22.6	500.9	1252.5
	2	31.8	505.8	1242.5
23	1	27.8	499.1	1224.0
	2	28.6	483.5	1292.0
26	1	31.1	499.4	1135.5
	2	29.0	503.7	1201.0
34	1	31.6	466.6	1176.0
	2	32.0	562.5	1291.5
Mean quality controls and assay variability				
Mean observed concentration		29.5	505.5	1240.2
Between-day error (R.S.D.) (%)		7.2	2.0	2.3
Within-day error (R.S.D.) (%)		10.7	4.8	3.8

VII. These data demonstrate the applicability of this assay in biopharmaceutic studies.

TABLE VI

CHROMATOGRAPHIC RETENTION TIMES FOR ETOPOSIDE, POTENTIAL DEGRADATION PRODUCTS AND/OR PRECURSORS

Compound	Retention time (min)
α-Ethylidene lignan P	3.9
4'-Demethyl-epipodophyllotoxin (Epi-aglycone)	4.8
cis-Hydroxy acid	6.2
Etoposide	8.5
Lignan P	8.9
Picro-ethylidene lignan P (picrolactone)	9.3
4'-Carbobenzoxy-ethylidene lignan P	N.D.*
4'-Carbobenzoxy-diformyl-ethylidene	N.D.
4'-Carbobenzoxy lignan P	N.D.

*N.D. = Not detected.

TABLE VII

PHARMACOKINETIC PARAMETERS OF DOG No. 4

TRT = treatment; C_{max} = maximum obtained plasma concentration; T_{max} = time at which C_{max} is reached; AUC = area under the plasma concentration—time curve; *h = theoretical hours (infinite); beta = rate constant governing the elimination of drug from plasma.

Pharmacokinetic parameter	TRT No. 1	TRT No. 2	TRT No. 3
C_{max} (ng/ml)	2648.11	992.67	1867.99
T_{max} (h)	0.3	0.75	0.5
AUC, 0—infinity (ng/ml *h)	2634.28	1779.19	2536.96
AUC, 0—*n* h	2367.68	1735.52	2375.4
No. of points used for beta	5	4	3
Correlation coefficient for beta	0.9603	0.9945	9.9959
Terminal half-life (h)	2.04	1.59	2.62

DISCUSSION

This assay was developed to analyze for etoposide in plasma to carry out pharmacokinetic studies of etoposide. Previous HPLC—UV methods did not provide adequate sensitivity for these studies [6] or required procedural modifications for detection of low levels of plasma etoposide [7]. While this assay was being developed, an HPLC—electrochemical detection assay was published [8]. This method had a limit of detection of 20 ng/ml for etoposide in plasma. The method described in this paper increases the sensitivity to 10 ng/ml.

The use of teniposide as an internal standard was investigated but was considered unnecessary due to the excellent linearity of standard curves without internal standard. Also, the use of an internal standard would have substantially increased analytical run times.

Etoposide shows good stability in the dissolution solvent. Over 48 h there is a small decrease in peak height; however, the stability data, along with the quality control data, indicate that the etoposide is stable enough so that all samples can be adequately analyzed during an overnight HPLC run. Plasma samples of etoposide are stable for at least one month at −30°C.

The detector response was reproducible over the several months involved in assay development and sample analysis. The porous graphite electrodes of the Model 5010 analytical cell were routinely flushed with 4 *M* nitric acid to remove residue absorbed from the plasma extracts. During the course of an analytical run, about 68 samples, the background current did show some increase, however, it did not affect the detector response.

This assay has provided the sensitivity necessary to obtain pharmacokinetic parameters following administration of etoposide to the dog. Based on the similarity of pharmacokinetics of man and the dog for many drugs, the same sensitivity will be necessary to determine pharmacokinetics in man. A standard curve in human plasma gave a correlation coefficient of 0.997 for a 10—1000 ng/ml concentration range. Quality control samples were accurate to within 10.5 and 6.3% for the theoretical concentrations 38.8 and 841.4 ng/ml with R.S.D. values of 10.4 and 4.9%, respectively. A human plasma assay would thus appear to be straightforward.

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