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Determination of unbound etoposide concentration in ultrafiltered plasma by high-performance liquid chromatography with fluorimetric detection

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Abstract

Etoposide is a highly protein bound drug, and monitoring the concentration of free drug could help individualize dosage in oncological patients. The cost and difficulty of the standard techniques (equilibration dialysis) has hampered the monitoring of free drugs. We describe a simple HPLC method for the measurement of free etoposide concentration in plasma. Sample preparation involves the ultrafiltration of plasma by a Centrifree device for 30 min at 2000 *g* and extraction with chloroform. The isocratic separation is performed with a μBondapak phenyl analytical column. Fluorimetric detection is used (288–328 nm excitation and emission wavelengths). Linearity of the calibration curve is excellent between 0.05 and 1 μg/ml. Accuracy and precision are reported at the concentrations 0.06 and 0.4 μg/ml: within-run accuracy is 10% and 6.2%, respectively; between-run accuracy is ≤1%; within-run coefficients of variation (C.V.) are 10.6 and 5.0%; between-run C.V. are 11.6 and 6.8% respectively. The range of the assay is 0.05 to 1 μg/ml. The feasibility of the technique has been tested in 7 patients treated with oral etoposide for hepatocarcinoma (mean protein binding 91%). We found no interference from endogenous substances, co-administered drugs (alizapride, furosemide, ranitidine) and other antineoplastic agents (doxorubicine, idarubicine, vinblastine, vinorelbine).

Keywords: Etoposide

1. Introduction

Etoposide (Fig. 1), an epipodophyllotoxine derivative, is a widely used antimitotic drug. Myelosuppression and antitumor activity correlate with systemic exposure to the drug. Recent evidence suggests that monitoring plasma concentrations of etoposide could help individualize etoposide dosage, reducing hematological toxicity while optimizing dose intensity [1]. One of the most striking characteristics of etoposide disposition is its high binding to plasma proteins (94% in plasma from healthy subjects) [2]. Protein binding is reduced in oncology patients; often, but not always, this reduced protein binding can be explained by a hypoalbuminemia or hyperbilirubinemia [3,4]. Since it is not the total, but rather the free drug concentration that correlates with the concentration at the site of action, the free drug concentration is often considered as the best quantitation of the pharmacologically active drug. For highly protein bound drugs, the free drug concentration is only a small percentage of the total

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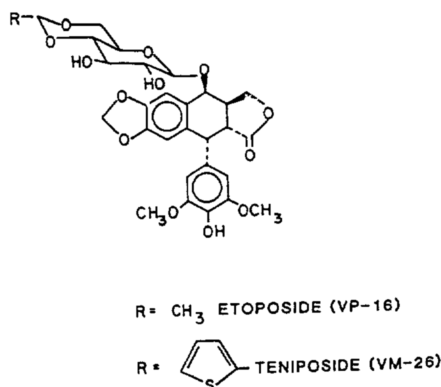

Fig. 1. Etoposide and teniposide chemical structure.

concentration. In the case of a decreased binding, the total etoposide concentration may underestimate the amount of active drug, and therapeutic and toxic effects will appear at lower total concentrations. Therefore, the systemic exposure to free drug may be the most useful pharmacokinetic parameter for pharmacodynamic studies and eventually therapeutic drug monitoring [4,5].

To establish dosage guidelines for patients in various pathological conditions, pharmacodynamic and dosage individualization studies including the measurement of the free concentration are needed. One study by Stewart et al. has shown that systemic exposure to free drug is a better predictor of myelosuppression than area under the total concentration–time curve [6]. However, probably because the classical technique of equilibrium dialysis with tritiated etoposide is costly and time-consuming, no other team has tackled the task of monitoring free etoposide concentration. We think that technical difficulties have hampered the clinical use of therapeutic drug monitoring of free etoposide. The separation of free drugs is now simplified by the availability of ultrafiltration devices. However, the etoposide concentration in the ultrafiltrate is often below the sensitivity of validated HPLC methods [7–10]. In this work we describe a validated method for the quantitation of plasma free etoposide concentration using ultrafiltration and a sensitive HPLC method with fluorimetric detection.

2. Experimental

2.1. Chemicals and reagents

Etoposide and teniposide (internal standard) were supplied by Bristol Myers (Rome, Italy). Stock solutions (0.1 mg/ml) were prepared in methanol and stored at −20°C. Methanol, chloroform and acetic acid were purchased from Carlo Erba (Milan, Italy), and acetonitrile from J.T. Baker (Deventer, The Netherlands). Distilled water was deionized by MilliQ water system from Millipore (Vimodrome, Italy).

2.2. Instrumentation

The HPLC system consisted of a Model 510 pump, a Model 717 plus autosampler, equipped with a 200-μl loop, a Model 470 scanning fluorescence detector and a Model 481 UV spectrophotometer from Waters (Milford, MA, USA). Data were acquired and processed with the Millenium 2010 chromatography manager software from Waters.

2.3. Chromatographic conditions

Separation was done using a μBondapak Phenyl analytical column (125 Å, 10 μm; 300×3.9 mm I.D.) from Waters and a mobile phase of acetonitrile–water–glacial acetic acid (35:64:1, v/v) at 1.0 ml/min. The excitation and emission wavelengths were set at 288 and 328 nm, respectively. A 60-min washout time was kept between 2 analyses to allow for elution of fluorescent compounds and return to an undisturbed baseline.

2.4. Ultrafiltration

Frozen plasma sample were rapidly thawed and vortex mixed for 10 s. Separation of free etoposide was done by ultrafiltration using the disposable Centrifree micropartition device from Amicon (Beverly, MA, USA). The molecular mass cut-off of the membrane is 30 000. A 1-ml plasma sample was divided in two aliquots. The ultrafiltration was performed for 30 min at 25°C in a 33° fixed angle centrifuge (Beckman Model G56R) running at 2000

g. Under these conditions, the ultrafiltration of 1 ml of plasma yields about 500 μl of ultrafiltrate.

The adsorption of etoposide on the membrane was studied at low (0.03 μg/ml), medium (0.2 μg/ml) and high (0.4 μg/ml) concentration. The aqueous solutions were ultrafiltered according to the described techniques. Etoposide concentration was measured in the solution (C_s) and in the ultrafiltrate (C_u). The fraction adsorbed (F_a) on membrane was calculated as: $F_a = 100 \cdot (C_s - C_u / C_s)$.

2.5. Extraction

The ultrafiltrate was transferred in a polyethylene tube, and spiked with internal standard (50 μl of a 0.01 mg/ml teniposide methanolic solution). Chloroform (1 ml) was added and slow agitation for 20 min was performed. After centrifugation (1000 g for 5 min) the aqueous phase was discarded by aspiration and the organic phase was evaporated to dryness by heating (40°C) under vacuum in a conical glass tube. The dry extract was rediluted in 50 μl methanol and 25 μl were injected.

2.6. Calibration curves

Quantitation was based on the internal standard method, using the ratio of peak areas and a calibration curve. Blank (drug free) ultrafiltrate was obtained by ultrafiltration of plasma from healthy blood donors. For the calibration curve 500 μl of blank ultrafiltrate were spiked with 0.5 μg of teniposide (I.S.) and 0.025, 0.05, 0.100, 0.250 and 0.500 μg of etoposide. The calibration curve and two quality control samples (0.06 and 0.4 μg/ ml) were run with every set of ten unknown samples. Quality control samples were run in triplicate on five different days to calculate within and between run accuracy and precision.

2.7. Determination of total plasma etoposide concentration

The total etoposide concentration (C_t) was determined by the technique described by Evans et al. [7] and modified by D'Incalci et al. [8]. In the modified method, UV absorbance detection was used instead of electrochemical detection. Briefly, the method can be described as follows. Each 1-ml plasma sample was spiked with I.S. (10 μg) and 8 ml of chloroform were added. After 20 min of slow agitation, 5 min centrifugation at 1000 g, the supernatant aqueous phase was discarded and the organic phase was evaporated to dryness. The dry residue was reconstituted in methanol (100 μl) and 25 μl were injected. Chromatographic conditions were similar to those described for the free fraction but the 60-min wash out period between samples was not necessary. The UV spectrophotometer was set at 254 nm. The calibration curve was constructed using a series of 5 plasma samples spiked with known amounts of etoposide (0.2, 0.5, 1.0, 5.0, 10.0 μg/ ml).

2.8. Clinical application

We studied seven patients with hepatocarcinoma treated daily with oral etoposide (100 mg soft gelatine capsules) and enrolled in a bioavailability study. C_t was measured at 1, 2, 4, 6 and 24 h after the oral dose. C_{max} and T_{max} (peak concentration and time of peak) were determined; C_f was determined in the sample with the peak concentration. Protein bound fraction (F_b) of etoposide was calculated as $F_b = 100 \cdot (1 - C_f / C_t)$.

2.9. Specificity

2.9.1. Endogenous substances

Blank plasma samples from healthy volunteers ($n=3$) and patients with hepatocarcinoma ($n=4$) were tested for interference with endogenous substances.

2.9.2. Drugs

We checked the charts of the patients to identify any concomitant therapy that could interfere with the fluorescence detection. We then tested the most commonly co-administered drug (ranitidine, furosemide and alizapride). Although our patients were treated by etoposide as a single antineoplastic agent, we investigated possible interference with other antineoplastic drugs (doxorubicin, idarubicin, vinblastine, vinorelbine). Aqueous solutions of each

drug (alone and with etoposide and I.S.) were injected directly into the HPLC system. Retention times (t_R) were compared and peak resolution was examined.

3. Results

3.1. Chromatograms

Fig. 2 illustrates the chromatograms obtained after extraction of the ultrafiltrate used for the measurement of free etoposide concentration (Fig. 2a, blank ultrafiltrated plasma; Fig. 2b ultrafiltrate of a sample drawn 2 h after oral administration of 100 mg of etoposide). The chromatograms obtained by the extraction of the same samples with the standard method (extraction of whole plasma and UV absorbance detection) are shown on Fig. 3. (Fig. 3a: blank plasma; Fig. 3b: peak etoposide concentration). Retention times were 6.5 and 18 min for etoposide and teniposide, respectively. The resolution from the solvent front and between etoposide and internal standard was satisfactory. Using fluorimetric detection, the limit of detection (i.e., the lowest concentration yielding a signal consistently three times above the baseline noise) was 10 times lower than with UV absorbance detection. (0.01 μg/ml and 0.1 μg/ml, respectively).

3.2. Adsorption on the ultrafiltration device

The fraction of etoposide adsorbed on the membrane (median and range) was 16.5% (11.2–20.1) at 0.03 μg/ml, 4.1% (1.8–8.0) at 0.2 μg/ml and 2.4% (1.6–3.8) at 0.4 μg/ml.

3.3. Extraction recovery

The mean extraction recovery of free etoposide was satisfactory (mean 76%; range 74%–81%) and constant in the range of the assay. Mean extraction recovery of teniposide was 78% (range 63–91%).

3.4. Validation data

The calibration curves were linear in the range of the assay and an excellent correlation coefficient (r) was consistently found over five different days (r= 0.998±0.00048 (mean±S.D.); $p \leq 0.0001$ using least square regression). The slope of the calibration curve was 1.096±0.033 (mean±S.D.; n=5). The inter-day

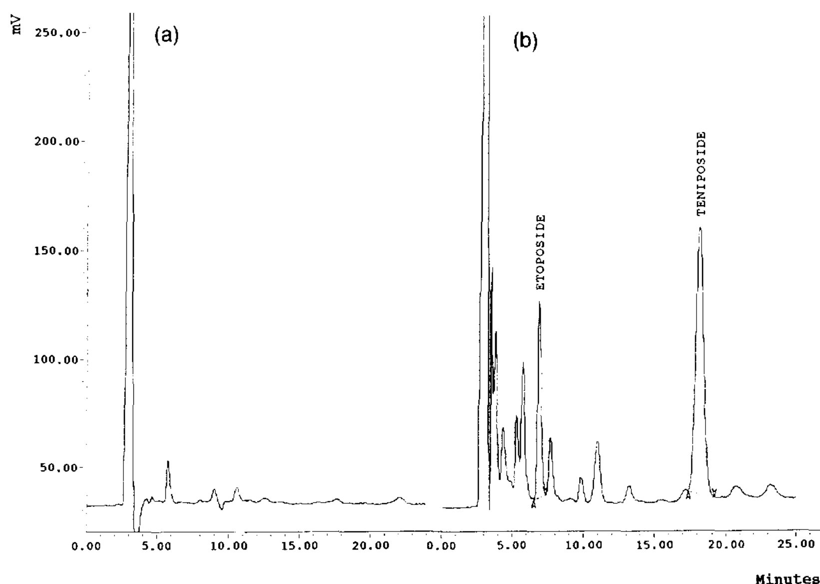

Fig. 2. Chromatograms obtained by extraction of plasma ultrafiltrate and fluorimetric detection: blank ultrafiltrate (a) and patient #7 ultrafiltrate (b; C_f=0.31 μg/ml).

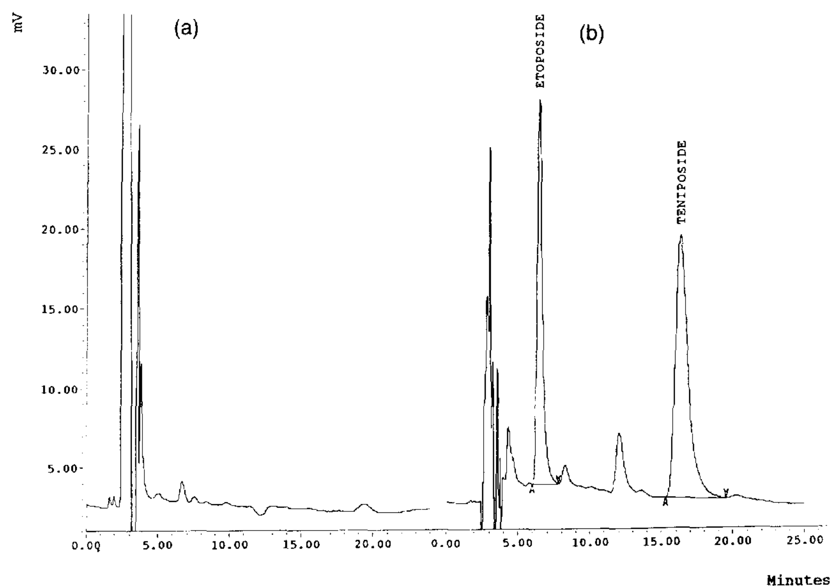

Fig. 3. Chromatograms obtained by extraction of whole plasma and UV absorbance detection (AUFS=0.01): blank plasma (a) and patient #7 peak concentration (b; C_t=5.3 μg/ml).

variability in the slope was 3.2%. Table 1 reports the theoretical and calculated concentrations for each point of the calibration curve in 5 different runs.

The results of the accuracy and precision (within-run and between-run) of the method are given in Table 2, and are all below 15%, which is an acceptable range for validated HPLC methods. The limit of quantitation (the lowest concentration that can be measured with acceptable accuracy and precision) was 0.05 μg/ml.

3.5. Clinical pharmacokinetics application

The peak plasma total concentration (C_{max}), free concentration (C_f) and protein bound fraction (F_b) in patients with hepatocarcinoma are reported in Table 3.

3.6. Specificity

In blank plasma samples, no interfering peak was found at the retention times of etoposide and teniposide. No endogenous substance from the plasma of healthy subjects or cancer patients interfered with the assay. Interference with co-administered drug was studied. There was no overlap between the peaks of the analytes [etoposide (t_R 7 min) and

Table 1
Theoretical and calculated concentrations for each point of the calibration curves in 5 different runs (mean and S.D.)

Theoretical concentration (μg/ml)	Calculated concentration (mean±S.D.) (μg/ml)
0.0500	0.0495±0.0072
0.100	0.102±0.0056
0.200	0.199±0.0060
0.500	0.498±0.0280
1.000	1.001±0.0120

Table 2
Within-run and between-run accuracy and precision (%C.V.) in the measurement of plasma etoposide unbound concentration

Spiked concentration (μg/ml)	Measured conc. (mean±S.D.)	Accuracy (%)	C.V. (%)
Within-run (n=5)			
0.060	0.066±0.007	10	10.6
0.400	0.375±0.002	6.2	5
Between-run (n=4)			
0.060	0.060±0.007	0	11.6
0.400	0.397±0.027	1	6.8

Table 3
Total and free plasma concentration (C_f) of etoposide observed in patients with hepatocarcinoma

Patient	Age (years)	Albumin (g/dl)	Bilirubin (mg/dl)	C_{max} (μg/ml)	C_f (μg/ml)	F_b (%)
1	83	3.3	1.2	4.9	0.42	91.5
2	58	4.8	0.5	6.6	0.43	93.5
3	65	3.9	4.4	1.4	0.21	84.4
4	70	3.5	0.6	6.1	0.64	89.5
5	68	3.5	1.5	3.5	0.28	92.2
6	74	4.0	1.0	3.7	0.21	94.3
7	61	3.4	2.8	5.3	0.31	94.1
Mean±S.D.	68±8	3.8±0.5	1.7±1.4	4.5±1.8	0.36±0.15	91.4±3.5

teniposide (t_R 17 min)] and the tested drugs: alizapride (t_R 6.0 min), furosemide (t_R 9.5 min), idarubicine and doxorubicine (t_R 21–22 min) and vinblastine (t_R 37 min); vinorelbine and ranitidine were not detectable under the analytical conditions used.

4. Discussion

Our objective was to develop a simple and economical method for monitoring the free etoposide concentration in plasma. The gold standard for the measurement of drug free fraction is the equilibration dialysis with the tritiated compound. This method has been used by a single team to measure protein binding of etoposide in various pathological conditions [3–6]. However, routine use of this method has been hampered by the costs of radioactive reagents and dialysis membranes, and the long time of analysis (each dialysis requires an equilibration time of at least 6 h). Disposable ultrafiltration devices are now available at low cost for the separation of the high-molecular-mass proteins. Adsorption of etoposide on the ultrafiltration device is negligible in the range of the assay. The molecular mass cut off of Centrifree membrane is 30 000, well above that of etoposide (588) and below the molecular mass of albumin and α-1acid-glycoprotein, the main plasma proteins which bind etoposide. The concentration of etoposide in the ultrafiltrate was expected to be below the sensitivity of standard HPLC methods [7–10]. We, therefore, had to develop a more sensitive method. This was done by optimizing extraction of the reference methods [7,8] and changing the detection method from UV absorbance to fluorimetric detection. Fluorescence was used by Strife et al. [9] with different chromatographic conditions, but the baseline noise was reportedly so high to require a subtraction of a blank plasma baseline from the chromatogram. Under our conditions, the baseline noise was not disturbing the quantitation of low concentration, provided a one hour wash-out time was allowed between analyses. This time could be shortened to 30 min by the use of a gradient of acetonitrile (data not shown). However, the isocratic technique described was found to be the best compromise between time of analysis and cost of equipment.

The relatively long time of chromatography (80 min per sample) seems acceptable, since a single sample per patient would have to be analysed by this method. In vitro assays have determined that % protein binding is constant for concentrations up to 10 μg/ml. Therefore a single determination of the free fraction in each patient seems sufficient to determine exposure to free drug. The extraction with chloroform from the ultrafiltrate was easier than from plasma, allowing a reduction in the solvent/matrix ratio and yielding a good extraction recovery. Because of the small amount of etoposide in the free fraction, 50% of the extract volume had to be injected. The limit of quantitation (the lowest concentration accurately measured with a coefficient of variation below 20%) is 0.05 μg/ml. (Tables 1 and 2). This method allows the HPLC measurement of unbound etoposide between 0.05 and 1 μg/ml; the free fraction can be measured between 2 and 30% of a total concentration in the range of peaks (2 to 5 μg/ml) observed with low dose (100 mg) oral

therapy. The clinically useful range is covered by this method, as confirmed by the preliminary clinical application. Protein binding of etoposide is reportedly 94% with normal subjects [2] and 86% in patients with cancer [3]. In our patients with hepatocarcinoma and various degree of liver dysfunction, we found a mean bound fraction in the expected range (91%) (Table 3).

Similar results have been found in oncological patients using the technique of equilibration dialysis [3–6]. In addition, we ruled out any interference from endogenous substance, co-administered drugs and some antineoplastic drugs that could be used in combination with etoposide. Our data confirm the clinical feasibility of an HPLC method for monitoring free etoposide concentration in oncological patients. This method should be applicable with no excessive cost in all laboratories equipped with HPLC.

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