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# Determination of etoposide in human plasma and leukemic cells by high-performance liquid chromatography with electrochemical detection

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**Abstract**

This paper describes a high-performance liquid chromatographic method with electrochemical detection for the determination of etoposide levels in plasma, total and non-protein bound concentration, and in leukemic cells. The precision for between-runs ($n=6$) was 7.0, 4.9, and 9.5%, the accuracy was 3.7, 7.1 and 6.3%, and within-runs precision ($n=6$) was 3.9, 2.9 and 5.1% for total plasma, non-protein bound plasma fraction and leukemic cells, respectively. The correlation coefficients ($R^2$) were 1.00 for all calibration curves. These assays have been applied to analyze samples from one patient with acute myelogenous leukemia during 24 h after i.v. infusion of etoposide (100 mg/m$^2$). 

*Keywords:* Etoposide

## 1. Introduction

Etoposide is a semi-synthetic glycoside derivative of podophyllotoxin. It is widely used in the treatment of various cancers [1–3]. Therapeutic drug monitoring of etoposide is not yet a routine. However, more studies aiming at the determination of therapeutic drug levels are warranted. To finish this task, it is necessary to use simple and sensitive methods for etoposide determination.

Etoposide has a high albumin-bound fraction in plasma (95%), and therefore the free-etoposide concentration is very low [4–6]. Small changes in the proportion of bound etoposide could have major implications for the unbound etoposide concentration. It has been shown that the non-protein bound or free etoposide concentration is more closely correlated to toxicity than the total etoposide concentration in plasma [7,8]. It has also been demonstrated that the etoposide concentrations are lower in solid tumors and leukemic cells than in plasma [9–11]. Therefore it is necessary to use highly sensitive methods to determine the drug levels in patient samples, especially when tumor cells and free etoposide concentrations in plasma are investigated.

Etoposide is a chiral drug and can be degraded

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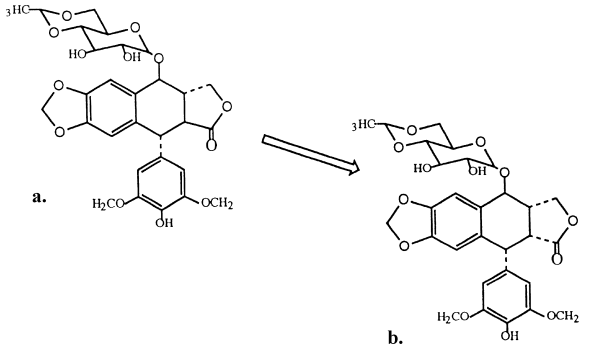


Fig. 1. The structures of *trans*- (a) and *cis*-etoposide (b).

from the *trans*-isomeric form of the lactone to the *cis*-lactone [12]. The structures of *trans*- (Fig. 1a) and *cis*-etoposide (Fig. 1b) are shown in Fig. 1. The stereo-chemistry of the *trans*-lactone is essential for cytotoxicity of etoposide, while the *cis*-isomer is inactive [13]. The *cis*-lactone is sometimes present in ultrafiltrates from plasma [9]. It is therefore important to separate the metabolite and the mother compound, particularly when free etoposide concentrations are determined.

High-performance liquid chromatography (HPLC) is one of the methods which is frequently used for drug analysis in pharmacokinetic studies [14,15]. One of the most important factors for sensitivity and accuracy in HPLC system is the choice of detection. The purpose of our study was to develop a sensitive and selective method for the determination of etoposide in human plasma and leukemic cells, using electrochemical detection.

## 2. Experimental

### *2.1. Chemicals and reagents*

Etoposide and the internal standard teniposide were kindly provided by Novartis Pharma Ltd. Etoposide solution 20 mg/ml, provided by the manufacturer Bristol-Myers Squibb, was also used. Acetonitrile, methanol, chloroform and acetic acid were all HPLC graded. Deionized water, filtered through a Millipore Milli-Q system (Millipore Corporation), was used throughout the performance.

### *2.2. Instrumentation and chromatographic conditions*

The HPLC system consisted of a Shimadzu pump LC-10AD (Shimadzu Corp., Kyoto, Japan), a Carnnegie CMA 200 injector (Carnnegie Medicine, Stockholm, Sweden) and a CSW Chromatography Station integrator (DataApex Ltd., Jinonice, Czech Republic). Detection was performed with a Coulochem II electrochemical detection (ESA Inc., MA, USA) equipped with a Model 5200A dual electrode analytical cell (electrode 1: +200 and electrode 2: +500 mV). A Model 5020 guard cell (+500 mV) was placed between the pump and the injector to oxidize any electroactive components in the mobile phase. The flow-rate of pre-filtered isocratic mobile phase, containing water–methanol–acetonitrile–acetic acid (52:43:4:1, v/v), was 1 ml/min. The stationary phase consisted of a Nucleosil® 7-μm (Phenomenex), 150×4.6 mm, as an analytical column, equipped with a NewGuard Phenyl precolumn.

### *2.3. Standard curves*

Total etoposide concentration in plasma: pooled plasma (0.5 ml/point) from healthy blood donors, obtained from the Blood Transfusion Center, Karolinska Hospital, was spiked with etoposide solution for an eight-point calibration curve at 0, 0.1, 0,5, 1, 5, 10, 20 and 30 μg/ml.

Free, non-protein bound etoposide concentration in plasma: calibration curves were prepared in water–methanol (50:50, v/v) solution (1 ml/point). The concentrations of the eight-point calibration curve were 0, 0.01, 0.025, 0.05, 0.1, 0.5, 1 and 2 μg/ml.

Cellular etoposide concentration: PBS buffer (pH 7.4) with BSA (40 μg/ml) (1 ml/point), spiked with etoposide at 0, 0.01, 0.025, 0.05, 0.1, 0.5, 1 and 2 μg/ml, was used for calibration of the cellular drug assay.

### *2.4. Within-run quality control samples*

Plasma from healthy blood donors, water–methanol (50:50, v/v) solution and PBS buffer with BSA (40 μg/ml), spiked with etoposide in three different concentrations, were used as quality control samples for each assay. These samples were prepared separ-

ately from the calibration samples and they were prepared from different stock solutions. The quality control samples for total plasma concentrations were 0.25, 2.5 and 25 μg/ml. For free etoposide concentration and cellular drug concentration, the quality control samples were 0.025, 0.1 and 1 μg/ml.

### 2.5. Sample extraction procedure

A liquid–liquid phase extraction procedure with chloroform was used for the extraction of etoposide from plasma [14]. The internal standard teniposide (100 μg/ml, 50 μl) was added to 0.5 ml plasma samples and then mixed with 2 ml chloroform. After shaking and centrifugation, the under phase was removed and evaporated under a stream of nitrogen gas at 37°C. The residue was re-dissolved in 1 ml water–methanol (50:50, v/v) solution and 25 μl were injected into the HPLC system.

Free etoposide was separated from total plasma by an ultrafiltration technique using a Millipore Centrifree® filter (Millipore Corporation) as described before [16]. After centrifugation, 50 μl ultrafiltrate was injected into HPLC system.

Sonicated leukemic cells suspended in 1 ml PBS were spiked with 20 μl teniposide at 100 μg/ml. After the extraction procedure, the residues were re-dissolved in 1 ml water–methanol (50:50, v/v) solution and the injection volume was 50 μl.

### 2.6. Validation of the assay

Between-runs ($n=6$) evaluation: The precision was calculated at each concentration of the standard curves, run at six occasions. The accuracy was defined as the percentage of deviation between the concentrations determined and their nominal value.

The accuracy was calculated as follows:

$$\frac{\text{Measured value} - \text{Nominal value}}{\text{Nominal value}} \times 100$$

The mean accuracy and precision of each assay was calculated based on all points of the standard curves.

Within-runs ($n=6$) evaluation: The precision of each assay was calculated based on the analysis of six samples run at three different concentrations of etoposide. The mean accuracy of each assay was also calculated, accordingly.

The limit of quantification (LOQ) was defined as the lowest concentration of the calibration curve, for which the accuracy and precision was validated.

### 2.7. Patient samples

Fresh peripheral blood samples were collected in heparinized glass tubes from a patient with AML. The patient was treated with a 1-h i.v. infusion of etoposide (100 mg/m$^2$) and subsequently blood samples ($n=8$) were taken during 24 h. Separation of plasma and isolation of leukemic cells were carried out according to previous description [17]. In brief, after centrifugation at 550 *g* for 5 min at 4°C, the plasma was separated. Leukemic cells were isolated by centrifugation on Lymphoprep®. Leukemic cell number and size were determined by using a Coulter Multisizer, and total cells volume of each sample was calculated. All samples were kept at −20°C until HPLC analysis.

One calibration curve was run together with the corresponding patient samples. Etoposide concentrations in plasma, total and free, and in leukemic cells were assayed on three separate days.

### 2.8. Calculation and statistics

Peak area ratios (etoposide/internal standard) were processed by a weighted linear regression. The slope and the intercept were calculated using Minim software on a Macintosh computer. The weighting function was $1/y$.

The concentration of the patient samples were calculated from the obtained calibration curve parameters of each assay.

The variables used for summary statistics performed on calibration and quality data include the arithmetic mean, the standard deviation, the precision and the accuracy.

## 3. Results

### 3.1. Chromatography

Fig. 2 shows a typical chromatogram from one patient plasma samples before (Fig. 2a) and after the etoposide infusion (Fig. 2b) in the mobile phase containing water–methanol–acetonitrile–acetic acid (52:43:4:1, v/v). The retention time for *trans*- and

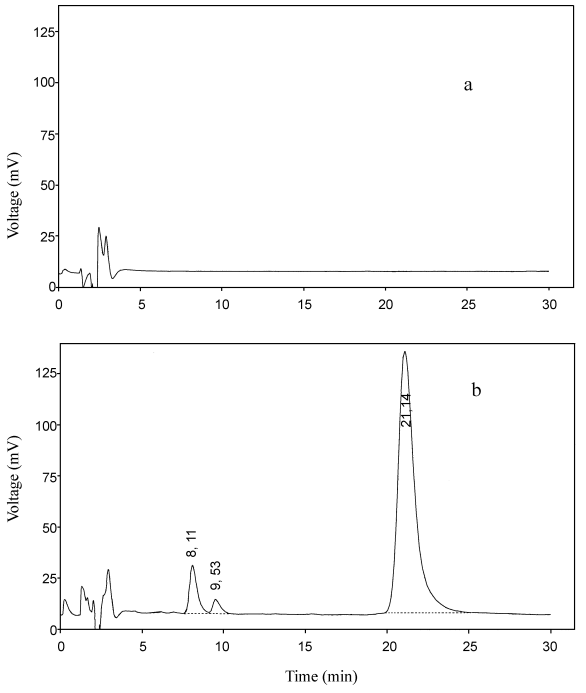


Fig. 2. Chromatograms of plasma samples from a patient before (a) and after (b) etoposide infusion. The retention times were 8.1 and 9.5 min for *trans*- and *cis*-etoposide, and 21.1 min for teniposide.

*cis*-etoposide was 8.1 and 9.5 min, respectively. Retention time for teniposide was 21.1 min. The analysis of the blank specimens (the samples before the addition of etoposide) for plasma, leukemic cells and free etoposide did not show any relevant interference at the retention time of neither etoposide nor teniposide.

### *3.2. Limits of quantification*

The limit of quantification of etoposide was 0.1 μg/ml for the total plasma assay, and 0.01 μg/ml for determination of free etoposide and leukemic cell concentration. In all cases, the precision and accuracy of the lowest points of the standard curves were ≤16 and ≤10%, respectively.

### *3.3. Precision and accuracy for between-runs and within-runs*

Table 1 summarizes the between-runs precision and accuracy for plasma, total and free fraction, and cellular drug assays. The mean accuracy of total plasma determination, free etoposide and leukemic cell assays was 3.7, 7.1 and 6.3%, respectively. The mean precision for total plasma, free etoposide and leukemic cell determinations was 7.0, 4.9 and 9.5%, respectively. The linearity of these assays in the selected concentration interval was $r^2 = 1.00$.

Table 1
Between-runs ($n = 6$), accuracy and precision of the etoposide assays

| Standard curves (μg/ml) | Accuracy (%) | Precision (%) |
|---|---|---|
| *Total plasma* | | |
| 0.1 | 5.5 | 8.9 |
| 0.5 | 0.7 | 7.3 |
| 1 | 6.5 | 4.1 |
| 5 | 1.4 | 9.4 |
| 10 | 3.8 | 9.6 |
| 20 | 4.0 | 8.4 |
| 30 | 3.9 | 1.3 |
| Mean value | 3.7 | 7.0 |
| *Free etoposide* | | |
| 0.01 | 10.0 | 15.9 |
| 0.025 | 10.7 | 2.1 |
| 0.05 | 11.0 | 4.1 |
| 0.1 | 11.3 | 2.8 |
| 0.5 | 4.6 | 5.1 |
| 1 | 0.4 | 2.7 |
| 2 | 1.9 | 1.9 |
| Mean value | 7.1 | 4.9 |
| *Leukemic cells* | | |
| 0.01 | 3.3 | 16.2 |
| 0.025 | 0.0 | 20.0 |
| 0.05 | 6.7 | 12.2 |
| 0.1 | 16.2 | 3.7 |
| 0.5 | 6.1 | 6.6 |
| 1 | 5.1 | 3.7 |
| 2 | 6.4 | 3.9 |
| Mean value | 6.3 | 9.5 |

Table 2 shows the within-runs precision. The mean precision for the double samples run at three different concentrations of the total and free etoposide plasma, and leukemic cell determinations were 3.9, 2.9 and 5.1%, respectively.

### *3.4. Analysis of patient samples*

Fig. 3 shows the etoposide concentration versus time profiles in plasma, total (Fig. 3a) and free etoposide concentration and cellular etoposide con-

Table 2
Within-runs ($n=6$) precision of the assays

| Control samples (μg/ml) | Precision (%) |
|---|---|
| *Total plasma* | |
| 0.25 | 3.6 |
| 2.5 | 2.3 |
| 25 | 5.8 |
| Mean value | 3.9 |
| *Free etoposide* | |
| 0.025 | 3.7 |
| 0.1 | 2.5 |
| 1 | 2.5 |
| Mean value | 2.9 |
| *Leukemic cells* | |
| 0.025 | 4.8 |
| 0.1 | 6.5 |
| 1 | 4.0 |
| Mean value | 5.1 |

centration (Fig. 3b) during 24 h from a patient receiving intravenous etoposide infusion at 100 mg/$m^2$ over 1 h. The results were expressed as μg etoposide per ml of plasma (μg/ml), ultrafiltrate or cell volume. No *cis*-etoposide peak was found in any of the samples from this patient. The free plasma fraction of etoposide varied from 1.4 to 2.8% of its total plasma concentration. The peak concentration was reached at the end of the etoposide infusion and declined according to the time profiles. The 24-h sample for cellular etoposide concentration was under the limit of quantification (0.01 μg/ml) and was considered as zero.

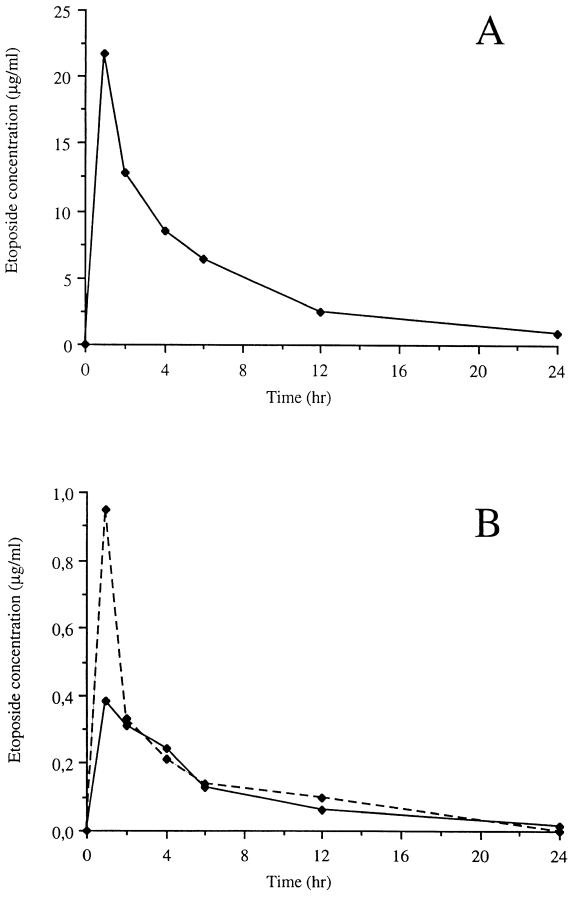


Fig. 3. Etoposide concentration versus time profiles in plasma, total (a) and free plasma fraction (– ♦ –) and in leukemic cells (- - ♦- -) (b) after 1 h i.v. infusion of etoposide (100 μg/$m^2$) from a patient with AML.

## 4. Discussion

Etoposide is one of the most commonly used anticancer drugs. Quantification of drug concentration in plasma, total and free fractions, and in tumor cells may provide important clinical information. Earlier report has described a method to analyze etoposide in patient plasma using reversed-phase HPLC with electrochemical detection [18]. Our paper here describes three assays for the determination of etoposide in human plasma, total and free plasma fraction, and in leukemic cells.

In previous HPLC assays for the determination of etoposide concentrations, mainly UV absorption [19–21] and fluorometric detection [22,23] have been used for detection. Lack of sensitivity is the main limitation of these methods. Etoposide plasma concentrations were easily determined with previously published methods. When free, non-protein bound, etoposide levels and cellular concentrations are assayed, the sensitivity of the method is crucial. Electrochemical detection is considered as the most selective and sensitive apparatus, therefore our HPLC assays are based on such detection.

In our assays, the lowest quantification limits for plasma, total and free fractions, and cellular etoposide concentration were 0.1, 0 01 and 0.01 μg/ml, respectively. The between-runs accuracy for the lowest quantification points were 5.5 and 10% in total and free plasma fractions, and 3.3% in leukemic

cells. The between-runs and within-runs precision of the lowest points were less than 16% and 4.8%. These results suggested that our methods were accurate and precise enough to determine the plasma and cellular etoposide levels in patient samples, even at very low levels. The range of the standard curves varied 200- to 300-fold with excellent linearity, suggesting that our assays are suitable to determine etoposide concentrations in plasma and cellular samples, even if they vary in a large range, which is often the case in pharmacokinetic studies.

Due to the high protein binding of etoposide, free drug concentrations can provide important information in pharmacokinetic studies. The free etoposide concentration in the patient samples ranged from 1.4 to 2.8% of total plasma drug concentration, which was in agreement with our earlier results [6,16]. Etoposide protein binding is interfered by many factors. Liu et al. [24] reported a strong negative correlation between serum albumin concentration and etoposide protein binding. Endogenous substances such as bilirubin and fatty acids will also interfere with etoposide protein binding by competing for binding sites on serum albumin or by altering the conformation of albumin [7,25]. Studies of protein binding have important implications for the pharmacodynamics of the drug. It has been shown that in hematological malignancies, free etoposide level was more related to drug toxicity than total etoposide level [7,8]. However, it still remains to be shown if this holds true concerning the drug efficacy. More studies addressing this issue are warranted. The lowest free etoposide concentration in the patient samples were 0.01 μg/ml at 24 h after i. v. etoposide infusion, which indicates that the limit of quantification of our assay was low enough for analysis of clinical samples. However, due to declining concentrations during the dose interval and decreasing cell numbers, the intracellular etoposide concentration was under the limit of quantification for patient cellular sample at 24 h post-infusion.

Our assays showed the capacity of measuring large concentration differences. The variation of etoposide concentration in the patient samples during 24 h treatment was 23- and 29-fold in total and free plasma fractions, respectively. The methods were rapid and simple to be performed, and they are considered suitable for pharmacokinetic studies in plasma and leukemic cells from patients receiving etoposide treatment at commonly used clinical dose.

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