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High-performance liquid chromatography with fluorometric detection for monitoring of etoposide and its *cis*-isomer in plasma and leukaemic cells

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Abstract

The podophyllotoxin derivative etoposide, extensively used in anticancer therapy, is highly protein-bound (95%) in plasma. It is a chiral drug and only the *trans*-isomer is pharmacologically active. Isomerisation to the inactive *cis*-lactone occurs in plasma. The *cis*-lactone is often present in ultrafiltrates of plasma from patients treated with etoposide, therefore it is important to separate the isomers when free etoposide concentrations are assayed. There is reason to believe that free and cellular concentrations are more important for the effect of etoposide therapy than total plasma concentrations. A high-performance liquid chromatographic (HPLC) method for quantification of etoposide and its *cis*-isomer in plasma, total and non-protein-bound concentrations, and in leukaemic cells is described. After addition of teniposide as internal standard the drugs were extracted with chloroform. Etoposide, its *cis*-isomer, teniposide and endogenous substances were separated isocratically on a Spherisorb phenyl reversed-phase column. Detection was performed fluorometrically, $\lambda_{ex\ em} = 230/330$ nm. Non-protein-bound concentrations were determined after ultrafiltration. The detection limit for etoposide was 10 ng/ml plasma, 25 ng/ml ultrafiltrate and 10 ng/$50 \cdot 10^6$ cells. The sensitivity of the assay for the *cis*-lactone was twice as high due to higher fluorescence. The protein binding of the *cis*-lactone in plasma from ten healthy blood donors was $54.5 \pm 4.8\%$ (mean ± S.D.). Thus, the free fraction was about ten-fold higher than that of the mother compound. The assay is convenient and sensitive enough for the determination of free and cellular fractions of etoposide.

1. Introduction

The podophyllotoxin derivative etoposide is extensively used in anticancer therapy, both of solid tumors and hematological malignancies [1–3]. Etoposide is a chiral drug and can be degraded from the *trans*-isomeric form of the lactone to the *cis*-lactone (Fig. 1). The stereochemistry of the *trans*-lactone is essential for cytotoxicity of etoposide [4].

There are large interindividual variations in the pharmacokinetic parameters of etoposide [5], which affects toxicity [6] and probably the therapeutic effect. Drug monitoring of etoposide for individualized dosing to optimize treatment is a future perspective.

Etoposide binds to a high extent (95%) to albumin in plasma [7] and the free concentra-

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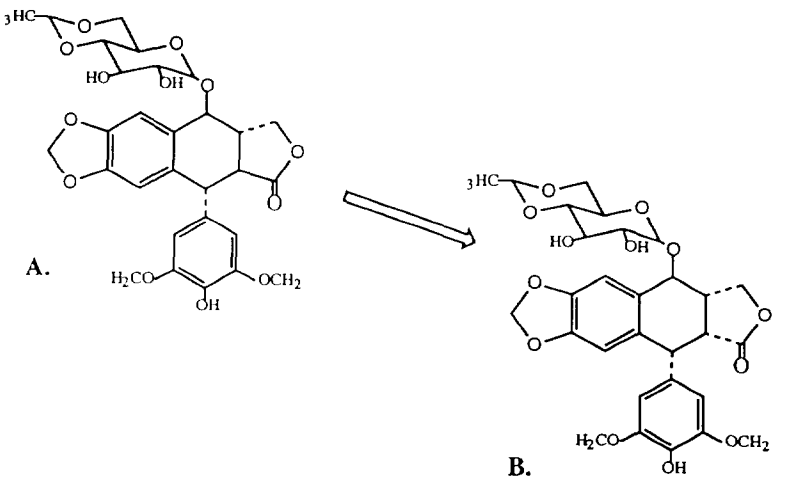

Fig. 1. Etoposide is degraded in neutral, weak acidic and alkaline conditions. The *trans*-lactone (A) is degraded through isomerization to the *cis*-lactone form (B), which is pharmacologically inactive.

tions after standard therapeutic doses are therefore low. It has been shown previously that non-protein-bound concentrations are more important for toxicity than the total etoposide concentrations in plasma during treatment [8]. The drug levels in solid tumor tissue and leukaemic cells are lower than the plasma concentrations [9–11]. Under these circumstances determination of non-protein-bound etoposide and cellular concentrations requires a sensitive drug assay. Furthermore, the *cis*-lactone is sometimes present in ultrafiltrates from plasma [11]. It is therefore important to separate the metabolite and the mother compound, particularly when free etoposide concentrations are determined.

A convenient high-performance liquid chromatographic method with fluorometric detection was developed for the quantification of etoposide and its *cis*-isomer in plasma, total and non-protein-bound concentrations, and in leukaemic cells.

2. Experimental

2.1. Chemicals

Etoposide and teniposide for analytical purposes were kindly provided by Bristol-Myers Squibb, Bromma, Sweden. The commercially available pharmaceutical formulations Vepesid and Vumon were also used. The *cis*-isomer of etoposide was synthesized according to the method previously described by Strife and co-workers [12]. Since they have equivalent molar extinction coefficients (pers. commun., Dr Rima B. Nassar, Bristol-Myers Squibb Pharmaceutical Research Institute), quantification of *cis*-etoposide was performed using a Hitachi U-2000 spectrophotometer assuming identical UV-absorbance at 200 nm of etoposide and the metabolite. All solvents used were HPLC grade.

2.2. Drug assay

Etoposide and its *cis*-isomer were determined by high-performance liquid chromatography (HPLC). Plasma samples (0.5 ml) and sonicated leukaemic cells ($50 \cdot 10^6$) in phosphate-buffered saline (PBS) (1 ml) were mixed with 2 ml of chloroform after addition of 2.5 μg teniposide to plasma and 0.5 μg to cell suspensions as internal standard. After evaporation of the organic phase under nitrogen, the residue was redissolved in 200 μl water–methanol (50:50). After sonication for 5 min 100 μl of the extract was injected into the HPLC system by a Carnegie CMA/200 refrigerated microsampler (Carnegie Medicine, Stockholm, Sweden).

A reversed-phase system with a Spherisorb Phenyl column 5 μm (250 × 4.6 mm) eluted with methanol–water–acetic acid (45:54:1) at a flow-

rate of 1.0 ml/min, was used to separate etoposide, *cis*-etoposide and teniposide from endogenous compounds.

Quantitation was performed fluorometrically exciting the compounds at 220 nm and observing the emission at 330 nm using a Shimadzu RF-535 Fluorescence HPLC Monitor. The signal was integrated using peak area ratios with a Macintosh computer program (ChroMac 354).

2.3. Determination of free drug concentrations

Non-protein-bound concentrations of the compounds were analyzed after removal of plasma proteins by ultrafiltration on Amicon Centrifree filters (W.R. Grace, Beverly, MA, USA) in 20°C. Subsequently 100–200 μl of the ultrafiltrate was injected into the HPLC system. Plasma from ten healthy blood donors was spiked with *cis*-etoposide, 1.0 μg/ml, and analyzed separately to determine the plasma protein binding of the metabolite.

2.4. Determination of cellular drug levels

The median cell volume and cell number were measured with a Coulter multisizer (Coulter Electronics, Luton, UK), and total cellular volume in the sample was calculated accordingly. The intracellular concentrations of etoposide and its *cis*-lactone were thereafter calculated by dividing the amount of drug measured with the total cell volume in each sample.

2.5. Calibration curves

Standard curves for calibration were prepared by spiking plasma or PBS supplemented with BSA (40 μg/ml) pH 7.4 with etoposide or *cis*-etoposide and run together with each set of samples. Plasma standard curves were used for determinations in plasma samples and buffer standard curves for determinations in cell samples. For determination of non-protein-bound concentrations an external standard curve with water–methanol (50:50) solutions of etoposide or *cis*-etoposide was used.

2.6. Validation of the assay

Pooled plasma from healthy blood donors and leukaemic cells from patients with acute myelocytic leukaemia were spiked with etoposide in the currently available pharmaceutical form Vepesid or newly synthesized *cis*-etoposide. The plasma concentrations were 1.0 and 5.0 μg/ml and the concentrations in cell samples were 1.0 and 0.1 μg/ml. For intra-assay variability eight samples were run consecutively. For day-to-day variation pooled plasma was spiked on day one, stored in −20°C and analyzed in duplicates or triplicates on five consecutive days thereafter.

2.7. Patient and sampling procedure

Samples were collected from one patient treated for acute myelocytic leukaemia, receiving etoposide 200 mg/m^2. The white blood cell count was $60 \cdot 10^9$/l and 70% were leukaemic blast cells. Peripheral blood samples were collected in heparinized glass tubes before, at the end of the 1 h infusion of etoposide and repeatedly thereafter during the first dose interval (24 h). The samples were immediately cooled in ice water and plasma was separated by centrifugation (550 *g*, 5 min, 4°C). The blood cells were suspended in ice-cold PBS and leukaemic cells were isolated by density gradient centrifugation on sodium metrizoate/Ficoll (Lymphoprep, Nycomed Pharma, Oslo, Norway), washed twice in cooled PBS and finally the cell pellet was suspended in 1 ml of PBS. Plasma and cells were stored at −20°C.

3. Results

Absorption and emission spectra of etoposide were run to select optimal conditions for fluorometry. Excitation at 230 nm and observation of the emission at 330 nm was chosen based on the absorption and emission maxima. By varying the methanol concentration of the mobile phase it was possible to separate the *cis*-isomer of

etoposide from the *trans*-isomer. It was also possible to obtain separation of teniposide from the *cis*-etoposide with reasonable retention times. Chromatograms showing a plasma sample before and after spiking with etoposide, *cis*-etoposide and teniposide, 2 μg/ml of each compound, are shown in Fig. 2. The retention times were 6.1 min for etoposide, 7.8 min for *cis*-etoposide and 9.3 min for teniposide. The limit of detection, defined as peak area three times larger than base line noise level, of etoposide was 10 ng/ml plasma, 25 ng/ml ultrafiltrate and 10 ng/$50 \cdot 10^6$ cells. The sensitivity of the assay for *cis*-etoposide was twice as high due to higher fluorescence of the metabolite.

The variability within and between assays for etoposide and *cis*-etoposide in plasma and cell samples is presented in Table 1. The studied concentrations of etoposide are in the range of plasma and leukaemic cell concentrations during treatment with etoposide.

Calibration curves were run in relevant ranges in plasma for plasma samples, in water–methanol solutions for ultrafiltrates and in PBS supplemented with BSA for cell samples (Table 2). All standard curves were linear and the coefficients of correlation were >0.998.

When water solutions of the *cis*- and *trans*-isomers of etoposide were chromatographed we initially obtained variable results and non-linear standard curves. Since these compounds are highly lipophilic, with a water solubility of etoposide of about 100 μg/ml at neutral pH, the drug sticks to glass surfaces. The addition of an organic solvent solved these problems. The amount of methanol in the injected solution affects the peak areas of etoposide and *cis*-etoposide in the chromatograms. The maximum peak area was reached when 40% methanol or more was used. Therefore water–methanol solutions (50:50) were always used for dilution of these compounds.

The free, non-protein-bound, fraction of *cis*-etoposide was determined after ultrafiltration of ten different plasma samples from healthy blood donors spiked with the metabolite 1 μg/ml. The protein binding of *cis*-etoposide was 54.5 ± 4.8% (mean ± S.D.).

The assay was used to delineate the pharmacokinetics in a patient with acute myelocytic leukaemia after 1 h i.v. infusion of etoposide 200 mg/m^2. The elimination curves of etoposide in plasma, total and unbound drug, along with the drug accumulation in peripheral leukaemic cells

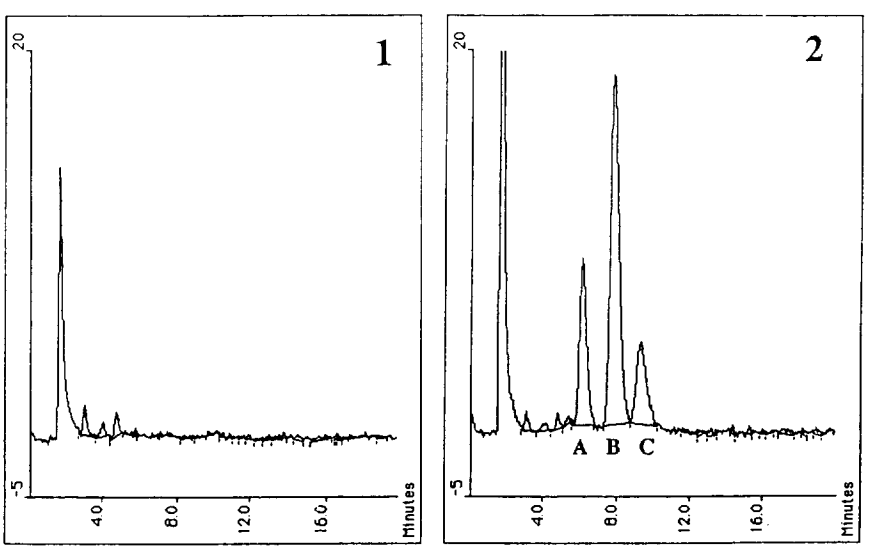

Fig. 2. Plasma chromatograms before (1) and after (2) addition of 2 μg/ml of (A) etoposide RT = 6.08 min, (B) *cis*-lactone of etoposide RT = 7.80 min, and (C) teniposide RT = 9.31 min. RT means retention time.

Table 1
Intra- and inter-assay variability of etoposide (*trans*-lactone) and its *cis*-isomer in plasma and leukaemic cell samples ($50 \cdot 10^6$ cells), and free fraction in ultrafiltrates of spiked plasma

	1.0 μg/ml	5.0 μg/ml
Plasma total concentration		
Etoposide intra-assay	7.1%	5.3%
between assays	4.5%	8.7%
cis-Lactone intra-assay	3.9%	5.4%
between assays	11.3%	5.6%
	5.0 μg/ml	10.0 μg/ml
Plasma free fraction		
Etoposide intra-assay	4.9%	7.4%
between assays	3.5%	8.9%
cis-Lactone intra-assay	3.7%	2.6%
between assys	6.9%	7.5%
	0.1 μg/sample	1.0 μg/sample
Cells		
Etoposide intra-assay	9.4%	5.0%
between assays	9.5%	6.0%
cis-Lactone intra assay	4.8%	3.4%
between assys	8.3%	4.3%

are shown in Fig. 3. Free etoposide was measurable throughout the 24 h and was 4.2 ± 1.1% of the plasma concentration. The cellular concentration was 14.9% of that in plasma at the end of the infusion. Due to low cellular concentrations of etoposide and decreasing cell numbers during treatment no drug was detected at 24 h. The *cis*-isomer was detected in the ultrafiltrates (Fig. 4). While the free etoposide concentration decreased from 1.73 μg/ml to 0.62 μg/ml, the *cis*-etoposide peak increased six-fold between the end of the 1 h infusion and 3 h post infusion.

4. Discussion

In previous HPLC assays for determination of etoposide concentrations UV absorption has mainly been used for detection [12–16]. Lack of sensitivity is the main limitation of such meth-

Table 2
Calibration curves used for the determination of etoposide (*trans*-lactone) and its *cis*-isomer in (1) plasma, (2) ultrafiltrates/non-protein-bound concentrations and (3) leukaemic cells

No.	Matrix	Compound (μg/ml)	Range (μg/ml)	r^2
1	Plasma	Etoposide	0.5–10.0	0.9995
	cis-Lactone	0.5–10.0	0.9988	
2	Water–methanol (50:50)	Etoposide	0.025–1.0	0.9995
	cis-Lactone	0.025–1.0	0.9986	
3	Buffer with BSA	Etoposide	0.05–1.0	0.9983
	cis-Lactone	0.05–1.0	0.9983	

BSA means bovine serum albumin.

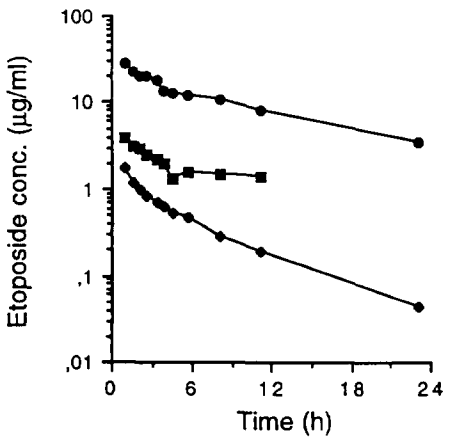

Fig. 3. Elimination curves of etoposide in plasma, total (●) and non-protein-bound (◆) drug, and in leukaemic cells (■) after 1 h i.v. infusion of 200 mg/m^2.

ods. Electrochemical detection increases the sensitivity [17,18]. Since the podophyllotoxins have a weak native fluorescence, fluorometric detection is possible. This reduces the considerable background noise from endogenous compounds in plasma and cells and is technically less complicated than electrochemical detection. Previously described methods using fluorometric detection lack sensitivity or specificity for the *trans*-isomer [19,20].

During an earlier investigation of intracellular etoposide concentrations in peripheral leukaemic cells from patients treated with etoposide, 100 or 200 mg/m^2, for acute myelocytic leukaemia, the need for method development was recognized since intracellular drug levels were significantly lower than plasma concentrations [11]. A reversed-phase HPLC system with a Nucleosil phenyl column and UV detection was previously used. Due to variable drug levels in leukaemic cells between patients, declining concentrations during the dose interval and sometimes low cellcounts, the sensitivity of that method was not sufficient for determinations of cellular etoposide levels in all patients.

In the same investigation we also studied free etoposide levels after ultrafiltration of the plasma samples. A *cis*-etoposide peak was sometimes found in the ultrafiltrates from patients treated with etoposide. The protein-binding properties

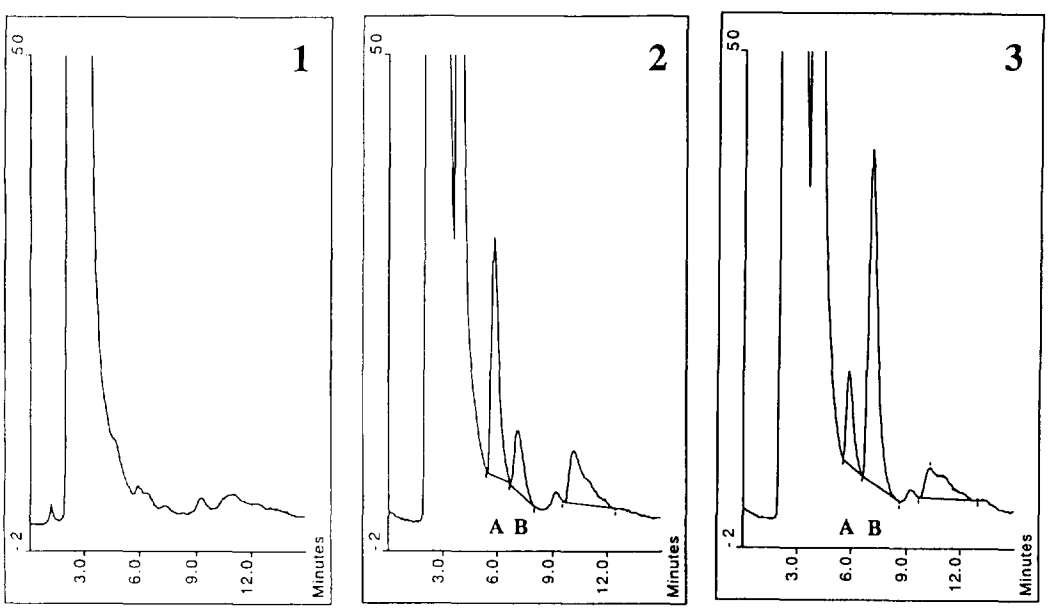

Fig. 4. Ultrafiltrate of patients plasma before the administration of etoposide (1) and at the end of 1 h infusion (2) of 200 mg/m^2. The concentration of unbound etoposide (A) was 1.73 µg /ml and of the *cis*-lactone of etoposide (B) 0.19 µg /ml. In the sample taken 3 h post infusion (3), the concentration of unbound etoposide (A) was 0.62 µg/ml and of the *cis*-lactone of etoposide (B) 1.18 µg/ml.

of the metabolite was therefore studied in plasma from healthy blood donors. The free fraction of *cis*-etoposide was 45.5% ($n = 10$). This is in contrast to etoposide where only 4–5% of the drug is unbound [7,21] and explains why it is detectable in the ultrafiltrates in spite of the low levels in plasma [5]. Because the *cis*-isomer is inactive, this finding emphasizes the need of chromatographic methods which separate the *cis*- and *trans*-isomers of etoposide when free drug concentrations are measured.

The described method was also used to study the pharmacokinetics of etoposide in a patient with acute myelocytic leukaemia receiving etoposide 200 mg/m^2 as 1 h infusion. The method presented is sensitive enough to allow the determination of total and free etoposide concentrations in plasma throughout a dose interval of 24 h after a standard dose. The *cis*-isomer was present in the ultrafiltrates. The cellular concentrations could be followed for 11 h in this patient. The assay can readily be used for therapeutic monitoring of etoposide in plasma and leukaemic cells.

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