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Determination of the cytotoxic catechol metabolite of etoposide (3′O-demethyletoposide) in human plasma by high-performance liquid chromatography

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Abstract

The anticancer drug etoposide undergoes O-demethylation in humans. The formed catechol metabolite exhibits significant cytotoxic activity. A simple, rapid, selective, and sensitive reversed-phase high-performance liquid chromatography assay was developed for the measurement of etoposide catechol in plasma of tumour patients. The metabolite was quantified using electrochemical detection. Ascorbic acid was added to each sample to prevent oxidation of etoposide catechol during sample preparation. Linear responses were obtained between 40 ng/ml and 1.25 μg/ml with correlation coefficients exceeding 0.991. The detection limit was 10 ng/ml. Recovery, within-day precision, between-day precision and accuracy were satisfactory. The method has been applied to characterize the concentration–time profile of etoposide catechol in plasma of tumour patients following administration of high-dose etoposide. © 1997 Elsevier Science B.V.

Keywords: Etoposide; 3′O-Demethyletoposide; Catechols

1. Introduction

The podophyllotoxin derivative etoposide is widely used in cancer chemotherapy [1,2]. Though the detailed mechanism of action of this drug has not been elucidated up to now its cytotoxic activity is mainly explained by the interaction with topoisomerase II inducing DNA strand breaks [3,4]. Moreover, there are several reports that oxidation of the phenolic group of etoposide leads to reactive intermediates which cause DNA damage [5,6]. In vitro investigations in rodent and human liver microsomes revealed that etoposide is O-demethylated by cytochrome P450 3A4 [7–9]. The formed metabolite 3′O-demethyletoposide (etoposide catechol) has been shown to exhibit considerable cytotoxic activity [10] and can be easily oxidized into the semi-quinone and *ortho*-quinone (Fig. 1). In contrast to parent etoposide, it directly inactivates biologically active DNA [11].

Because of the potential contribution of the catechol to antitumour activity and toxicity of etoposide future pharmacokinetic studies should include the determination of the catechol metabolite. Relling et al. [12] measured etoposide catechol concentrations in urine of children after etoposide treatment. In order to characterize the plasma kinetics of the metabolite we developed a high-performance liquid chromatography (HPLC) method for its quantification in plasma of tumour patients.

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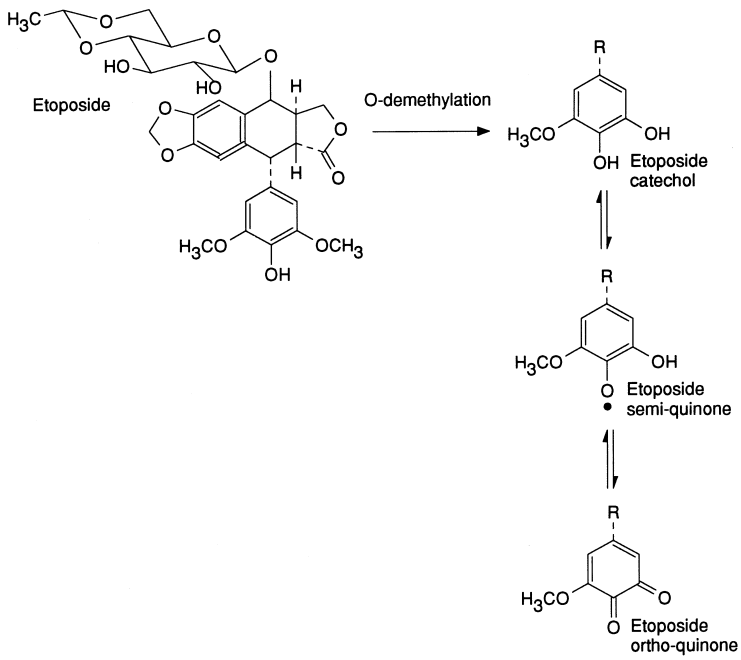

Fig. 1. O-Demethylation and subsequent oxidation of etoposide.

For etoposide several rather sensitive HPLC assays are described using electrochemical detection [13–16]. This mode of detection resulted in higher sensitivity and selectivity compared to UV absorption [16]. Since plasma concentrations of the metabolite were expected to be lower than those of etoposide, electrochemical detection was chosen.

In this paper we describe the first analytical assay for measuring etoposide catechol in plasma for use in clinical pharmacokinetic trials.

2. Experimental

2.1. Chemicals and solvents

3′O-Demethyletoposide was synthesized by mono O-demethylation of etoposide [17] and kindly supplied by Dr. R. Kasper, Department of Pharmaceutical Biology, Freie Universität Berlin. Its identity was verified by NMR and mass spectroscopy. It was purified using column chromatography.

All organic solvents used were HPLC grade (Promochem, Wesel, Germany). Water for HPLC was generated by a Milli-Q water preparation system (Millipore, Eschborn, Germany). Disodium hydrogenphosphate analytical-reagent grade was purchased from Merck (Darmstadt, Germany) and ascorbic acid analytical-reagent grade from Sigma (Deisenhofen, Germany).

2.2. Instrumentation

The chromatographic system consisted of a Model 125 programmable solvent module as the pump, a Model 507 automatic injector 507 and a Model 406 analogue interface module (all Beckman Instruments, Munich, Germany). Electrochemical detection was carried out using an ESA-Coulochem II 5200 A detector with a Model 5010 dual electrode analytical cell (Bischoff, Leonberg, Germany). A Model 5020 guard cell (Bischoff) with an operating voltage of 700 mV was installed between pump and injector to purify the mobile phase of potentially interfering electroactive components. The system was controlled using System Gold software (Beckman Instruments).

2.3. Chromatographic conditions

A Hypersil ODS RP-18, 5 μm, 120×4 mm, column (Knauer, Berlin, Germany) was used as the stationary phase. The mobile phase consisted of a mixture of methanol–0.01 *M* disodium hydrogenphosphate (43:57, v/v). After combining all constituents (apparent) pH was adjusted to 6.0 with phosphoric acid. Flow-rate was 0.4 ml/min. The mobile phase was continuously degassed using a Model DG-1210 degasser (VDS Optilab, Berlin, Germany).

2.4. Sample preparation

Twenty μl of an aqueous solution of ascorbic acid (1 mg/ml) were added to 150 μl of plasma to avoid oxidation of etoposide catechol during sample preparation. In order to precipitate plasma proteins 300 μl acetonitrile were mixed with each plasma sample followed by centrifugation at 3200 *g* for 10 min. Subsequently, the supernatant was evaporated to dryness using a Speed Vac plus SC 110A evaporator (Savant Instruments, Farmingdale, NY, USA). The residue was reconstituted in 150 μl of mobile phase, and 20 μl of this solution were injected onto the HPLC system.

2.5. Sample stability

Sample stability during the analytical procedure was assessed by multiple regular injections of a spiked plasma sample containing 1 μg/ml etoposide catechol stored at room temperature for different periods of time. For comparison, aliquots of the same sample were frozen at approximately −24°C at the beginning of the experiment. At predetermined times, one aliquot was thawed 5 min before injection and analyzed immediately before the sample kept at room temperature was measured. Stability was calculated using the following equation:

$$\text{Stability (\%)} = \frac{\text{Peak height of the sample stored at room temperature} \cdot 100}{\text{Peak height of the sample stored at } -24^{\circ}\text{C}} \quad (1)$$

2.6. Preparation of stock and calibration solutions

A stock solution of etoposide catechol (607.41 μg/ml) and ascorbic acid (1 mg/ml) was prepared in methanol–water (3:1, v/v) and stored at approximately −70°C. Blank plasma was spiked with various volumes of etoposide catechol stock solution to obtain nine calibration solutions with concentrations ranging from 20 ng/ml to 5.0 μg/ml. The calibration curve was checked for linearity by linear regression, aliquoted and stored at approximately −24°C. One aliquot of each calibration solution was analyzed with each run.

2.7. Assay validation

Limit of detection was defined as the peak height which was three-times larger than baseline noise. Limit of quantification was defined as the lowest calibration solution which could be measured with a precision below 20% and an accuracy between 80 and 120%.

Recovery was determined in triplicate at 0.05, 0.3, 1.0 and 5.0 μg/ml by comparing the peak height of spiked plasma samples with that of corresponding aqueous solutions that were treated like plasma samples.

Accuracy was calculated by relating the measured concentration of two spiked plasma samples to their nominal concentration as determined by weighing (0.26 and 2.43 μg/ml).

Within-day variability was assessed by ten consecutive injections of plasma samples spiked with four different concentrations of etoposide catechol (0.05, 0.3, 1.0 and 5.0 μg/ml).

Between-day variability was assessed using two spiked and four biologically derived plasma samples (controls). The biological controls were obtained by pooling residual plasma of patients receiving etoposide. An aliquot of each sample was analyzed with each run.

3. Results

3.1. HPLC separation and method optimisation

In order to find out the optimum potential for electrochemical detection of etoposide catechol we recorded the voltammogram of the compound in mobile phase. The peak height which is proportional to the electric current was measured for the same sample at different potentials (Fig. 2). Maximum

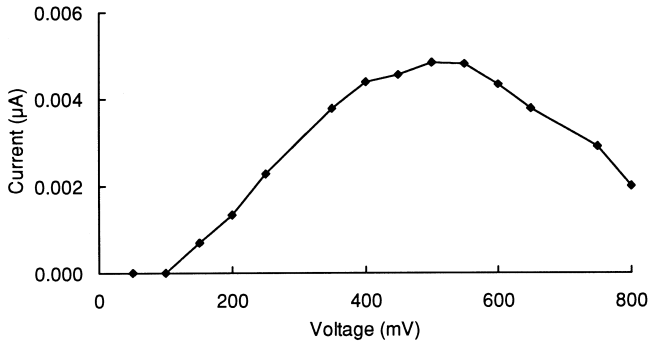

Fig. 2. Voltammogram of etoposide catechol.

oxidation was reached between 450 to 550 mV. The first electrode of the dual electrode cell was used to increase selectivity by screening out electroactive compounds which oxidized at or below the set potential, and the second electrode was used for detection. Since according to the voltammogram a potential of 100 mV does not affect the oxidation of etoposide catechol this potential was chosen for the first electrode. The second electrode was set to 450 mV in order to maximize oxidation of the catechol and to minimize oxidation of interfering compounds from plasma.

Fig. 3 shows two chromatograms obtained before and 3.5 h after administration of etoposide to a tumour patient. Using the selected conditions etoposide catechol can be selectively measured in plasma with a retention time of 9.0 min. Etoposide eluted much later with a retention time of about 15.2 min. No interfering peak was observed.

3.2. Sample stability

We evaluated the stability of etoposide catechol in prepared plasma samples over a period of 24 h with and without adding ascorbic acid as antioxidant (Fig. 4). Without ascorbic acid the peak height of etoposide catechol showed an unacceptable variability and decreased with time. When ascorbic acid was added etoposide catechol was stable for the examined period of time and variability was considerably reduced.

3.3. Linearity and sensitivity

The use of peak heights led to better results than peak areas. Calibration graphs using peak heights had a mean slope of 212.6±27.4 (S.D.) nA·l/mg and a mean intercept of −2.8±1.8 (S.D.) nA using six data points (40 ng/ml to 1.25 μg/ml). Coefficients

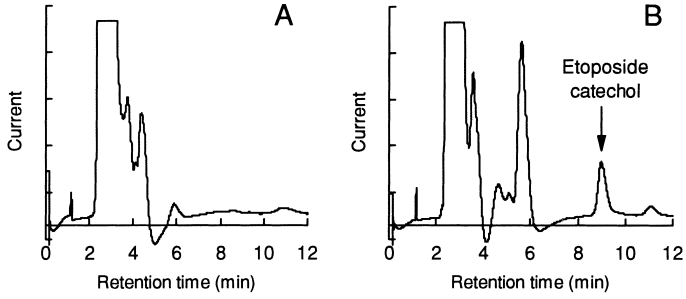

Fig. 3. Representative plasma chromatograms (A) before infusion of etoposide and (B) 3.5 h after the end of infusion of etoposide (measured concentration of etoposide catechol: 1.1 μg/ml).

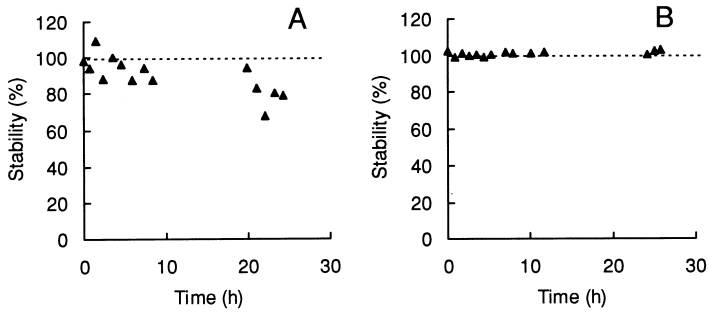

Fig. 4. Sample stability of etoposide catechol in plasma (A) without addition of ascorbic acid and (B) with addition of ascorbic acid.

of correlation ranged between 0.991 and 0.999. Limit of quantification was found to be 40 ng/ml, limit of detection was 10 ng/ml.

3.4. Recovery, precision and accuracy

Recovery of etoposide catechol with this assay was found to be 90.1±10.2% for 5.0 μg/ml, 82.5±5.4% for 1.0 μg/ml, 81.1±4.2% for 0.3 μg/ml, and 83.6±6.9% for 0.05 μg/ml, respectively. Precision data are summarized in Table 1. Within-day precision was about 3% and concentration independent. Between-day variation was performed by analyzing one aliquot of the same plasma sample on ten different days. Spiked and biological controls gave similar results. The coefficients of variation ranged from 8.3 to 15.1%. Accuracy was calculated using spiked controls of known concentration and was found to be 103.0±8.6% for 2.43 μg/ml (spiked control 1) and 100.5±10.7% for 0.26 μg/ml (spiked control 2).

3.5. Application of the assay

The applicability of the described method was demonstrated in a pharmacokinetic trial with tumour patients. Serial blood samples were collected from ten patients with germ cell cancer receiving etoposide in a high-dose chemotherapy regimen. Fig. 5 shows the mean concentration–time profile of etoposide catechol and parent etoposide after infusion of etoposide over 1 h in a dose of 600 mg/m^2. Peak concentrations of etoposide catechol ranged between 0.8 and 2.1 μg/ml. In all patients etoposide

Table 1
Within-day and between-day precision of the described assay

	Control sample	Concentration (μg/ml)	Coefficient of variation (%)
Within-day precision	1	5.0	2.9
	2	1.0	3.1
	3	0.3	3.0
	4	0.05	4.3
Between-day precision	Spiked control 1	2.43	8.3
	Spiked control 2	0.26	10.7
	Biological control 1	0.65[a]	8.7
	Biological control 2	0.68[a]	9.3
	Biological control 3	0.25[a]	15.1
	Biological control 4	0.11[a]	8.9

[a]Mean value of all measurements.

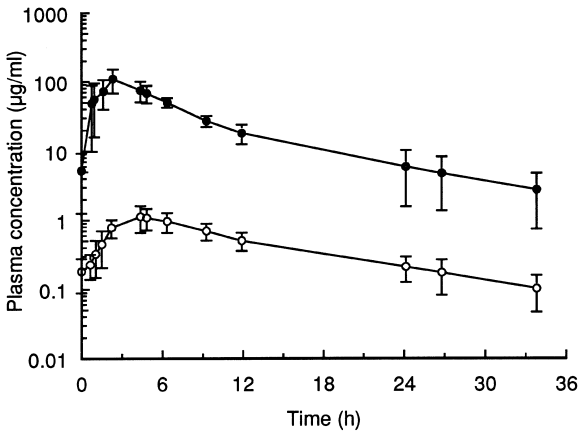

Fig. 5. Mean plasma concentrations (±standard deviations) of etoposide catechol (○) and parent etoposide (●) in ten patients after intravenous infusion of 600 mg/m^2 etoposide. Parent etoposide was measured using a validated reversed-phase HPLC method with UV detection and teniposide as internal standard (modified from [18]).

catechol could be measured up to 34 h after the end of infusion.

4. Discussion

Etoposide catechol can be quantified in plasma of tumour patients using reversed-phase HPLC with electrochemical detection. The stability tests revealed that the catechol must be protected from oxidation by addition of ascorbic acid before sample preparation. Since a similar stabilization is not necessary for parent etoposide the catechol structure must be responsible for this instability. Under the influence of oxygen the catechol can be oxidized to the *ortho*-quinone [11] which might be prevented by ascorbic acid. The observed high variability of peak height without addition of ascorbic acid could be explained by the different contact time with oxygen during sample preparation. In order to avoid any instability in our stock solution we dissolved etoposide catechol together with an excess of ascorbic acid.

Although there are no experimental data it can be assumed that in plasma etoposide catechol is strongly bound to proteins as known for etoposide [19]. With the described sample preparation we recovered more than 80% of the metabolite suggesting that the catechol is sufficiently released from proteins by acetonitrile precipitation. Recovery tended to be higher at 5.0 μg/ml compared to lower concentrations.

We did not find an appropriate internal standard for our method. Nevertheless, a sufficient within-day precision of 3 to 4% was achieved. Between-day precision was about 10% for most control samples. The reason for the relatively high coefficient of variation of 15.1% for one biological control (0.25 μg/ml) is unclear. Ruggedness of our method was satisfactory since results were not affected by different operators, different reagent lots or different elapsed assay times.

Our method has been applied to measure plasma levels of etoposide catechol in ten patients receiving high-dose etoposide. It was found to be sensitive enough to describe a full concentration–time profile of etoposide catechol in high-dose chemotherapy.

The described method can be extended to achieve simultaneous determination of etoposide and etoposide catechol. However, run time would be considerably prolonged since etoposide elutes much later than the catechol due to its higher lipophilicity. Moreover, both compounds cannot be quantified with electrochemical detection due to the large concentration difference. A solution is serial coupling of a UV detector for etoposide and an electrochemical detector for catechol measurement. First experience in our laboratory demonstrates the feasibility of this approach.

In conclusion, the present HPLC assay permits the simple, rapid, selective and sensitive quantification of etoposide catechol in plasma. The applicability of the method has been demonstrated in a pharmacokinetic trial in tumour patients receiving high-dose etoposide.

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