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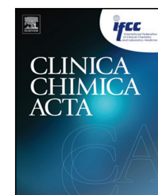

A simple dried blood spot method for clinical pharmacological analyses of etoposide in cancer patients using liquid chromatography and fluorescence detection

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ABSTRACT

Background: Therapeutic drug monitoring of etoposide is not part of the routine clinical practice, however, measuring etoposide plasma concentration may be useful to prevent chemotherapy related adverse drug reactions. This paper describes the development and validation of a dried blood spot (DBS) assay for the determination of etoposide in blood samples of lung cancer patients.

Methods: The whole blood spot was cut out of the DBS card followed by sonication assisted liquid drug extraction. Extraction solution was evaporated and re-dissolved. A high-performance-liquid-chromatography method with fluorimetric detection ($\lambda_{ex} = 230$ nm; $\lambda_{em} = 330$ nm) was used.

Results: Method met the validation criteria in terms of selectivity, linearity (0.5–20.0 μg/mL), accuracy (≥96.1%), precision (≤10.1%) and stability (long term 4 weeks at room temperature and 40 °C). Haematocrit did not influence DBS etoposide concentration. Good correlation between measured plasma and DBS concentrations was observed. The equation considering only haematocrit value was used for conversion of DBS to plasma concentration.

Conclusions: DBS sampling method showed comparable results to plasma samples. Therefore, it can be concluded that the developed and validated DBS method, which is more patient-friendly and requires less sample handling, is a reliable alternative to conventional plasma methods for measuring etoposide concentration in clinical pharmacological analyses.

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1. Introduction

Etoposide (Fig. 1) is an antineoplastic drug, a derivate of podophyllotoxin, which was granted Food and Drug Administration approval more than 30 years ago [1]. Its mechanism of action is formation of ternary complex with topoisomerase II enzyme and deoxyribonucleic acid (DNA) and prevention of resealing the broken DNA that normally follows topoisomerase binding to DNA. This leads to an accumulation of DNA breaks and cell death. It has significant therapeutic activity in childhood leukaemia, Hodgkin's disease, testicular tumours, large cell lymphomas and small cell lung cancer (SCLC) [2].

As for a majority of cytotoxic drugs, therapeutic drug monitoring (TDM) of etoposide is not part of the routine clinical practice. However, there are large intra- and inter-individual variations in the pharmacokinetic parameters of etoposide, which potentially result in suboptimal tumour cytotoxicity or excess toxicity, especially following oral etoposide administration [3]. As observed in a recently published observational study, advanced SCLC patients treated with standard etoposide/platinum regimen according to current guidelines, frequently developed high-grade neutropenia or febrile neutropenia, a serious toxicity of chemotherapeutic agents. Furthermore, the association between severity of neutropenic events and etoposide peak plasma concentration has been well perceived; concentrations were the highest in patients with febrile neutropenia and declined to the lowest levels observed in patients without neutropenia. To better predict post-chemotherapy neutropenic events in SCLC patients on etoposide and platinum treatment and to tailor therapy with granulocyte colony-stimulating factors, which are not unambiguously indicated, in a most efficient way, measuring etoposide plasma concentration might be of great value [4].

A number of analytical approaches using HPLC [5–15], UPLC-MS [16] and LC-MS [17,18] have been reported for assay of this drug. The reported HPLC methods have used electrochemical (ECD) [5,9,11], fluorescence (FLD) [6–8,13], ultraviolet [10,12–15] or visible [14] detection. In general, these methods use a reverse-phase column with an organic

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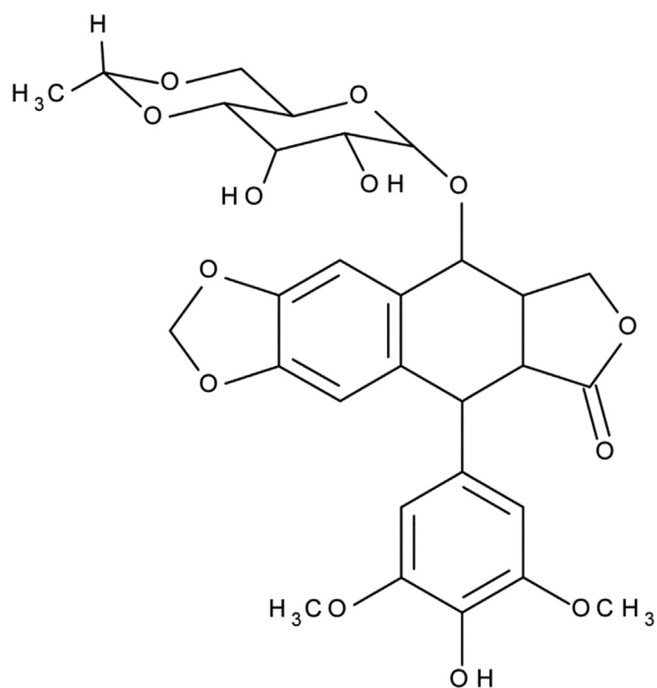

Fig. 1. Chemical structure of etoposide.

solvent (acetonitrile and/or methanol) and water or buffer-containing mobile phase. The most commonly used internal standard has been teniposide [5–7,11,18]. Additionally reported internal standards have been podophyllotoxin [8], phenacetin [10], diphenylhydantoin [12] and lamotrigine [14]. All cited analytical methods carry out sample preparation. This has variously been accomplished using liquid–liquid extraction with chloroform [5–7,11,15,17], ether [10], dichloromethane [12], acetonitrile [18] or mixture of chloroform and methanol [9] or n-hexane [14]. Alternatively, solid phase extraction has been described by few researchers [8,13,16]. The most common biological sample was plasma [5–7,9–18], rarely serum [8,17].

The collection of plasma or serum samples is the current standard for etoposide determination, while drops of whole blood collected on filter paper represent friendlier alternative of sample collection. Advantages of dried blood spots (DBS) sampling include minimally-invasive (finger stick, small volume of blood) and low cost (cheap supplies, collection of samples by non-medically trained persons at patient's home) sample collection as well as minimal requirements for sample processing (application of blood to filter paper, drying, storage and transportation in gas impermeable plastic bags) [19]. Moreover, DBS sampling is particularly appreciated in specific populations as are new-borns, pregnant women or critically ill patients where ethical concerns propose collection of smaller volume of blood samples.

Our objective was to develop and validate a method for determination of etoposide in whole blood samples using the DBS. Method was used for determination of etoposide blood concentration in advanced SCLC patients included in the pharmacokinetic study. Furthermore, results obtained by DBS assay were compared to etoposide plasma concentrations of the same patients obtained by conventional method using venipuncture blood collection. To the best of our knowledge this is the first method describing etoposide analysis in the DBS samples.

2. Materials and methods

2.1. Chemicals and materials

Etoposide and teniposide (internal standard for plasma samples) were purchased from Sequoia Research Products (Pangbourne, United Kingdom). N-dansyl-L-threonine cyclohexylammonium salt as an internal standard (IS) for DBS samples, methanol (for HPLC, ≥99.9%), acetonytrile (for HPLC, ≥99.9%) and Minipax® absorbent packets were purchased from Sigma-Aldrich (Steinhein, Germany). Ultrapure water was obtained from apparatus Milli-Q Advantage A10 Ultrapure Water Purification System, Millipore (Bedford, USA). Orthophosphoric acid, potassium dihydrogen phosphate and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Whatman 903 Protein Saver Cards for blood spotting were obtained from GE Healthcare (Dassel, Germany).

2.2. Etoposide determination in DBS

2.2.1. Standard stock, working and calibration solutions

A stock solution of etoposide (1.5 mg/mL) was prepared by dissolving etoposide in methanol. Etoposide working solutions for spiking blood were prepared from stock solutions by dilution with methanol to produce desired etoposide concentrations. Spiked blood calibration standards were prepared by spiking 240 μL of drug-free ethylenediaminetetraacetic acid (EDTA) anticoagulated fresh blood with 10 μL of the working solutions to yield blood etoposide concentrations of 0, 0.5, 1, 2, 3, 6, 9, 12, 15, 20, 25 and 30 μg/mL. Equally, low, medium and high quality control (QC) samples were prepared and obtained concentrations were 1.5, 5 and 17.5 μg/mL, respectively.

DBS calibration standards and QC samples were prepared weekly. A volume of 20 μL of each calibration standard and QC sample was spotted directly onto the DBS card. Blood spots were allowed to dry on air for at least 1 h then inserted in sachets of low gas permeability with absorbent packets and stored at room temperature. Preliminary experiments revealed that DBS samples were stable for at least 1 week.

A stock solution of the IS (1 mg/mL) was prepared by dissolving N-dansyl-L-threonine in an appropriate volume of methanol-acetonitrile-water (25:25:50; *v*/*v*/v). The stock solution of IS was diluted with the same solvent as used for dissolving IS to produce final concentration 0.06 mg/mL.

2.2.2. Patient sampling

The venous blood samples were collected from advanced SCLC patients during 4 chemotherapy cycles with etoposide/platinum on days 1, 2 and 3 of each cycle. The blood count and haematocrit level were measured before chemotherapy administration in each patient. Maximum 3 samples were collected each day of a course, i.e. 3, 6 and 24 h after etoposide administration. The blood was collected in Vacutainer tubes containing potassium EDTA. 20 μL of anticoagulated blood was spotted onto the DBS card and processed according to procedure for calibration standards as described above.

The remaining patients' blood was used for preparation of plasma samples. The blood was immediately centrifuged at 1500 × g and 4 °C for 10 min and stored at −80 °C until analysis. The study was approved by the Slovenian Ethics Committee.

2.2.3. Dried blood spot extraction

The whole blood spot was cut out of the DBS card and transferred to a microcentrifugation tube. A 750 μL of the extraction solvent consisting of methanol, acetonitrile and water (35:35:30, *v*/*v*/v) and 20 μL of 0.06 mg/mL IS solution were added to microcentrifugation tube. Samples were vortexed for 30 s, sonicated for 5 min at room temperature and then centrifuged for 5 min at 11,000 ×g. 650 μL of extraction solution was evaporated to dryness under the nitrogen stream and redissolved in 100 μL mixture of methanol and water (1:1, *v*/*v*). Samples were then centrifuged for 10 min at 5 °C and 16,000 ×g and injected into the HPLC-FLD system.

2.2.4. Chromatographic conditions

The chromatographic analyses were performed on Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany). The system consisted

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