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Rapid and sensitive liquid chromatography-tandem mass spectrometry method for determination of protein-free pro-drug treosulfan and its biologically active monoepoxy-transformer in plasma and brain tissue

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ABSTRACT

For the first time a high performance liquid chromatography method with tandem mass spectrometry detection (HPLC-MS/MS) was developed for simultaneous determination of a pro-drug treosulfan (TREO) and its active monoepoxide (S,S-EBDM) in biological matrices. Small volumes of rat plasma (50 μL) and the brain homogenate supernatant (100 μL), equivalent to 0.02 g of brain tissue, were required for the analysis. Protein-free TREO, S,S-EBDM and acetaminophen, internal standard (IS), were isolated from the samples by ultrafiltration. Complete resolution of the analytes and the IS was accomplished on Zorbax Eclipse column using an isocratic elution with a mobile phase composed of ammonium formate – formic acid buffer pH 4.0 and acetonitrile. Detection was performed on a triple-quadrupole MS via multiple-reaction-monitoring following electrospray ionization. The developed method was fully validated according to the current guidelines of the European Medicines Agency. Calibration curves were linear in ranges: TREO 0.2–5720 μM and S,S-EBDM 0.9–175 μM for plasma, and TREO 0.2–29 μM and S,S-EBDM 0.4–44 μM for the brain homogenate supernatant. The limits of quantitation of TREO and S,S-EBDM in the studied matrices were much lower in comparison to the previously used bioanalytical methods. The HPLC-MS/MS method was adequately precise (coefficient of variation $\leq$ 12.2%), accurate (relative error $\leq$ 8.6%), and provided no carry-over, acceptable matrix effect as well as dilution integrity. The analytes were stable in acidified plasma and the brain homogenate supernatant samples for 4 h at room temperature, for 4 months at −80 °C as well as within two cycles of freezing and thawing, and demonstrated 18–24 h autosampler stability. The validated method enabled determination of low concentrations of TREO and S,S-EBDM in incurred brain samples of the rats treated with TREO, which constitutes a novel bioanalytical application.

1. Introduction

Since the end of the 20th century alkylating agent treosulfan (TREO) has been applied in treatment of advanced ovarian carcinoma [1]. Nowadays the drug is frequently used for myeloablative conditioning prior to hematopoietic stem cell transplantation (HSCT). Based on a number of successful clinical phase I/II trials conducted in adult and pediatric transplant patients the drug has been allowed to enter a pivotal randomized phase III clinical trial which is supposed to decide on the marketing authorization for its use prior to HSCT [2,3]. From pharmacological point of view, TREO is an untypical pro-drug because it undergoes a non-enzymatic sequential epoxy-transformation in the human body to biologically active species, *i.e.* (2S,3S)-1,2-epoxybutane-3,4-diol-4-methanesulfonate (S,S-EBDM) and finally (2S,3S)-1,2:3,4-diepoxybutane (S,S-DEB) (Fig. 1) [4,5]. Pharmacokinetics of TREO is generally known based on the monitoring of its levels in the patients' plasma and urine, but the data on its biologically active forms are scarce as only plasma S,S-EBDM concentration profiles have been reported so far in two pediatric patients [6–9]. Moreover, till now no studies have been conducted to determine levels of either TREO or its biologically active epoxides in tissues, including central nervous system (CNS). Meanwhile, one of the target groups of patients for TREO-based therapies are children and older adults who are particularly susceptible to penetration of drugs into brain tissue because of deficiencies of the biological barriers [2,10–12]. The mentioned lack of the pharmacokinetic data probably stems from the difficulties in quantitative

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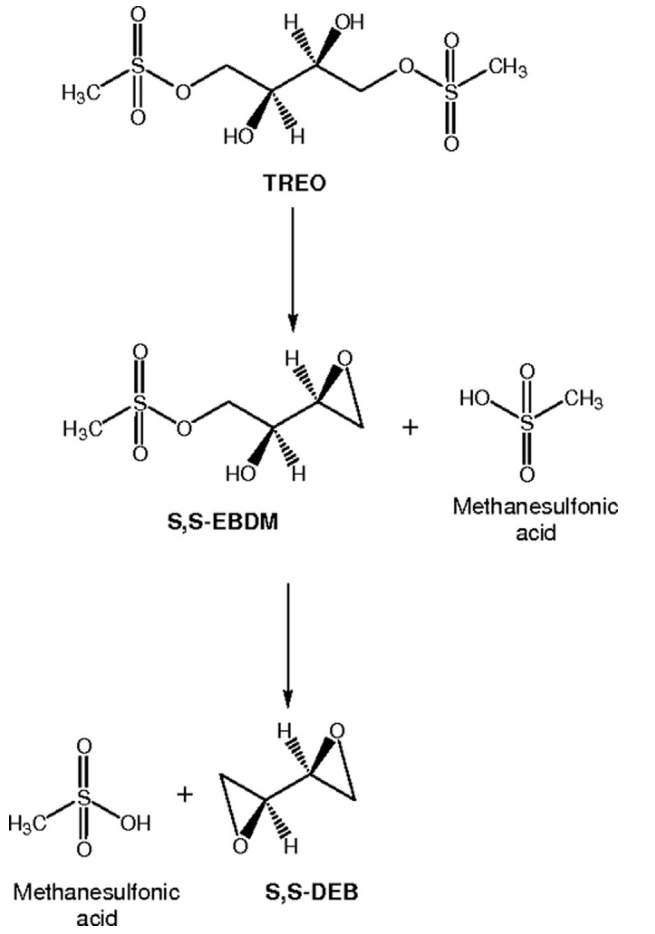

Fig. 1. Non-enzymatic transformation of TREO to its epoxides with alkylating activity.

bioanalysis of TREO and, especially, its epoxides. Neither TREO nor its epoxides possess chromophores (Fig. 1), therefore they cannot be directly analyzed using a popular HPLC method with UV or more sensitive fluorescence detection. TREO is administered in much higher doses than other drugs, *i.e.* 3–8 g/m^2 in ovarian carcinoma patients and 10–14 g/m^2 in conditioning prior to HSCT. For this reason an HPLC method with universal but low sensitive refractive index detection (RID) is successfully applied to assay of TREO in the clinical plasma and urine samples. Depending on the publication and the injection volumes (100 or 200 μL) the lower limit of quantitation (LLOQ) of TREO in plasma ranged from 3.6 to 36 μM, while a typical C_{max} of the drug met in the patients' plasma reaches up to 1.5 mM [2,6,13]. Recently, the HPLC-RID technique has been also developed for simultaneous determination of TREO and both its epoxy-transformers in the phosphate buffer and the LLOQ values were found to be 20 – 50 μM [14]. However, due to the poor sensitivity and selectivity, the RID does not offer a valid determination of low concentrations of S,S-EBDM and S,S-DEB in human plasma as well as expected low levels of TREO, and particularly its epoxides in such complex matrix as the brain tissue [9]. The indirect HPLC-UV method based on derivatization of an epoxide ring with 3-nitrobenzenesulfonic acid provides simultaneous quantitation of S,S-EBDM and S,S-DEB in human plasma with the LLOQ 2.5 μM, but it is laborious, time-consuming and does not enable determination of TREO itself [9]. Meanwhile, pharmacokinetic studies require the analysis of numerous samples collected at different time-points, hence the optimal methodology should provide fast determination of the analytes. Gas chromatography with mass spectrometry detection seems optimal for sensitive and selective analysis of S,S-DEB in various biological matrices, but offers no possibility to analyze TREO and S,S-EBDM because of their non-volatile nature [15–19]. In this paper we describe a novel rapid and sensitive HLPC method with tandem mass spectrometry detection (HPLC-MS/MS) for determination of TREO and S,S-EBDM in plasma and brain tissue. The method was successfully applied to quantitative analysis of the both analytes in the incurred samples from the rats treated with an intravenous bolus of TREO.

2. Material and methods

2.1. Materials

A certified standard of TREO was kindly supplied by medac GmbH (Wedel, Germany). Acetaminophen, used as an internal standard (IS), ammonium formate and formic acid were purchased from Sigma-Aldrich (St. Louis, USA). Sodium hydroxide volumetric solution 0.1 M (± 0.1%) and citric acid, both of analytical grade, were obtained from P.O.Ch. (Gliwice, Poland). Acetonitrile, HPLC gradient grade, was purchased from Merck KGaA (Darmstadt, Germany). Demineralized water at a conductivity of 0.1 μS/cm, prepared in a deionizer Simplicity UV (Millipore, USA) and filtered through a 0.45 μm cellulose membrane filter (Sartorius, Germany), was always used. The drug-free rat plasma and the drug-free brain tissue were obtained from Laboratory of Pharmacology and Toxicology (Hamburg, Germany).

2.2. Standard solutions of TREO and S,S-EBDM

Since a reference standard of S,S-EBDM is not commercially available, the compound was obtained by alkalization of TREO aqueous solution with equimolar amount of NaOH, according to the procedure specified in [14]. Briefly, 0.1392 g of TREO was dissolved in 15 mL of water in a 25 mL volumetric flask, titrated with 5 mL of 0.1 M NaOH volumetric solution and filled up with water to 25 mL. The obtained stock solution contained approximately 5 mM TREO, 10 mM S,S-EBDM and 5 mM S,S-DEB (molar ratio 1:2:1). The identity of S,S-EBDM was confirmed on the basis of the mass spectra registered with the HPLC-MS method. Real concentrations of S,S-EBDM in the solution (8.73 mM) was established as a difference between the initial concentration of TREO (20 mM) and a sum of the real concentrations of TREO (5.72 mM) and S,S-DEB (5.55 mM) after the alkalization with NaOH, quantified by the HPLC-RID method [14]. The standard solutions were prepared by diluting the appropriate volume of the TREO and S,S-EBDM stock solution with water in 10 mL volumetric flasks (Table 1). All the solutions of TREO and S,S-EBDM were freshly prepared each time before the analysis because of the limited stability of the epoxide.

2.3. HPLC – MS/MS conditions

Determination of TREO and S,S-EBDM was carried out in a chromatograph Agilent 1200 coupled to a triple-quadrupole mass spectrometer model 6410B Triple Quad with an electrospray (ESI) interface (both from Agilent Technologies, USA). The HPLC system consisted of a binary pump set at a flow rate of 0.4 mL min^{-1}, a solvent degasser, an autosampler and a thermostated column compartment. The separation was accomplished at 40 °C in a Zorbax Eclipse Plus C18 column (2.1 × 100 mm; 3.5 μm particle size) (Agilent Technologies, USA) guarded by an on-line filter. The mobile phase was composed of 0.01 M ammonium formate – formic acid buffer pH 4.0 and acetonitrile (95:5, v/v). Before the application to the HPLC system, it was always de-aerated using an