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Determination of prodrug treosulfan and its biologically active monoepoxide in rat plasma, liver, lungs, kidneys, muscle, and brain by HPLC–ESI–MS/MS method

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ABSTRACT

A prodrug treosulfan (TREO) is currently investigated in clinical trials for conditioning prior to hematopoietic stem cell transplantation. Bioanalysis of TREO and its active derivatives, monoepoxide (S,S-EBDM) and diepoxide, in plasma and urine underlay the pharmacokinetic studies of these compounds but cannot explain an organ pharmacological action or toxicity. Recently, distribution of TREO and S,S-EBDM into brain, cerebrospinal fluid, and aqueous humor of the eye has been investigated in animal models and the obtained results presented clinical relevance. In this paper, a selective and rapid HPLC–ESI–MS/MS method was elaborated and validated for the studies of disposition of TREO and S,S-EBDM in rat plasma, liver, lungs, kidneys, muscle, and brain. The two analytes and codeine, internal standard (IS), were isolated from 50 μL of plasma and 100 μL of supernatants of the tissues homogenates using ultrafiltration Amicon vials. Chromatographic resolution was accomplished on C18 column with isocratic elution. The limits of quantitation of TREO and S,S-EBDM in the studied matrices ranged from 0.11 to 0.93 μM. The HPLC–MS/MS method was adequately precise and accurate within and between runs. The IS-normalized matrix effect differed among the tissues and was the most pronounced in a liver homogenate supernatant (approximately 0.55 for TREO and 0.35 for S,S-EBDM). Stability of the analytes in experimental samples was also established. The validated method for the first time enabled determination of TREO and S,S-EBDM in the six life-important tissues in rats following administration of the prodrug.

1. Introduction

Treosulfan (TREO), a structural analog of busulfan, is registered in some European countries for treatment of advanced ovarian cancer [1]. Moreover, for the last decade TREO has been more and more widely applied as a myeloablative agent prior to hematopoietic stem cell transplantation (HSCT) in adult and pediatric patients in phase I/II clinical trials [2–8]. Currently, a randomized phase III clinical trial that compares TREO-based conditioning therapy with busulfan-based reduced-intensity conditioning is ongoing [9]. TREO is a prodrug that converts to biologically active derivatives, (2*S*,3*S*)-1,2-epoxybutane-3,4-diol-4-methanesulfonate (S,S-EBDM) and (2*S*,3*S*)-1,2:3,4-diepoxybutane (S,S-DEB), in a non-enzymatic reaction [10–12]. The knowledge of pharmacokinetics of TREO, S,S-EBDM and S,S-DEB originates mainly from the analyses of these compounds in human or animal plasma and the prodrug levels in human urine, in which an HPLC with UV, refractometric (RID) or MS/MS detection were used [6,12–18]. A few studies have reported biodistribution of TREO and its epoxy-transformers into other tissues/fluids, that is brain and cerebrospinal fluid of rats, and aqueous humor of the rabbit eye [19–21]. For the simultaneous quantification of TREO and S,S-EBDM in the rat central nervous system, an HPLC–MS/MS method with an electrospray ionization (ESI) was developed [19]. Both compounds were found to poorly penetrate across the blood – brain barrier, though the brain exposure was significantly greater in juvenile rats when compared to young adult ones. This may, at least in part,

Abbreviations: CV, coefficient of variation; EMA, European Medicines Agency; ESI, electrospray ionization; HSCT, hematopoietic stem cell transplantation; IS, internal standard; LLOQ, lower limit of quantitation; MF, matrix effect; RID, refractive index detector; r.t., room temperature; SD, standard deviation; S,S-DEB, (2S,3S)-1,2:3,4-diepoxybutane; S,S-EBDM, (2S,3S)-1,2-epoxybutane-3,4-diol 4-methanesulfonate; TREO, treosulfan.

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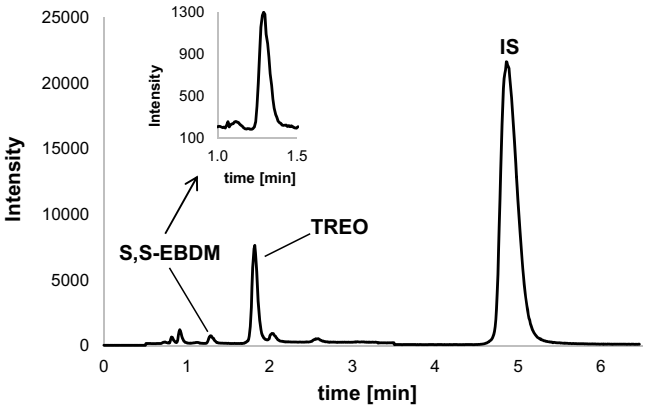

Fig 1. Representative total ion current chromatogram obtained during the analysis of the quality control sample of the liver homogenate supernatant spiked with 55 μM TREO and 94 μM S,S-EBDM. The inset graph shows an enlargement of the peak of S,S-EBDM.

relate to the occurrence of seizures in some infants treated with high-dose TREO prior to HSCT [2,20]. Moreover, a poor penetration of TREO across the blood – aqueous humor barrier in the rabbit eye corresponds to rather unsatisfactory results of the systemic treatment of uveal melanoma [21]. The levels of TREO and its epoxides in the other organs have not been investigated so far. However, this analysis is warranted as it will contribute to a comprehensive examination of the distribution of the two compounds in the body and better understanding of relatively low organ toxicity of the TREO-based conditioning. Therefore, the aim of the present study was to elaborate an HPLC–ESI–MS/MS method for the quantitative analysis of TREO and S,S-EBDM in rat liver, lungs, kidneys, femoral muscle, brain, and plasma, to validate the method, and confirm its usefulness in real *in vivo* setting.

2. Materials and methods

2.1. Materials

TREO was supplied by Medac GmbH (Hamburg, Germany). Formic acid, ammonium formate and codeine (internal standard, IS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade citric acid was purchased from P.O.Ch. (Gliwice, Poland). HPLC grade acetonitrile was obtained from Merck KGaA (Darmstadt, Germany). The water with a conductivity of 0.1 μS/cm was prepared in a deionizer Simplicity UV (Millipore, USA) and filtered through a 0.45 μm cellulose membrane filter (Sartorius, Germany). Due to the fact that S,S-EBDM is not commercially available it was freshly prepared before each analysis by titrating a TREO aqueous solution with an equimolar amount of NaOH, as described elsewhere [22].

2.2. HPLC–MS/MS conditions

Determination of TREO and S,S-EBDM was carried out using an Agilent chromatograph LC1200 combined with a triple-quadrupole mass spectrometer model 6410 B Triple Quad (Agilent Technologies, USA). The separation of compounds was accomplished at 40 °C on a Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 3.5 μm) with a guard column (Agilent Technologies, USA). The mobile phase was composed of ammonium formate – formic acid buffer (0.01 M; pH 4.0) and acetonitrile (95:5, v/v). The flow rate of the mobile phase was set to 0.4 mL/min. The injection volume was 5 μL. The eluent from the column was introduced directly to the ESI interface, which operated in positive mode. Nitrogen was used for nebulization (40 psi; 275.8 kPa) and as a drying gas with a flow rate of 10 L/min at 300 °C. The analytes were detected in multiple reaction monitoring (MRM) mode using the following mass transitions: TREO 296.0 → 279.1 (5 eV), **296.0 → 183.1** (5 eV), 296.0 → 87.1 (9 eV); S,S-EBDM **200.1 → 87.1** (5 eV); and codeine 300.0 → 215.0 (25 eV), **300.0 → 165.0** (45 eV) (transitions used for quantification are shown in bold and collision energies are given in the brackets). The monitored parent ions corresponded to the adducts of TREO and S,S-EBDM with ammonium cation (*m*/*z* 296.0 and 200.1, respectively) and a protonated codeine (*m*/*z* 300.0). The *m*/*z* 296.0/279.1 transition was supposed to result from a loss of neutral ammonia molecule from the parent ion of TREO. The mass transitions of *m*/*z* 296.0/183.1 (TREO) and *m*/*z* 200.1/87.1 (S,S-EBDM) were assumed to originate from the cleavage of ammonium methanesulfonate from the parent ions of the analytes. Moreover, the daughter ion of TREO with *m*/*z* 183.1 presumably underwent further fragmentation by a loss of neutral methanesulfonic acid molecule, which gives rise to the 296.0/87.1 mass transition. A fragmentor voltage for monitoring of TREO and S,S-EBDM was 50 V and for codeine it was 130 V. The system operation and data acquisition was controlled by the Mass Hunter software (Agilent Technologies, USA).

2.3. Preparation of calibration standards for determination of TREO and S,S-EBDM in plasma

The calibration standards were prepared as described elsewhere unless otherwise stated [19]. Briefly, blank rat plasma was spiked with standard solutions of the analytes and the IS (codeine), and filtered through Amicon vials (cut-off 30 kDa). The obtained filtrate was diluted with 0.25 mM aqueous solution of citric acid and injected into the HPLC–MS/MS system. Two ranges of calibration curve were prepared for quantification of TREO in plasma. The calibration curve I covered concentrations of 0.22–110 μM and the calibration curve II included concentrations of 110–5522 μM. The calibration standards falling into the TREO calibration curve II were more diluted during preparation when compared with the calibration curve I standards (250 and 6 times, respectively). This procedure prevented overloading of the MS/MS system and suppression of the TREO signal, therefore enabled to obtain a linear plot of the calibration curve in the whole range of the analyte concentrations. A calibration curve for S,S-EBDM ranged from 0.93 to 187 μM.

2.4. Preparation of calibration standards for determination of TREO and S,S-EBDM in tissue homogenate supernatants

In order to determine TREO in rat liver, lung, kidney, and muscle tissue homogenate supernatants also two independent calibration curves were prepared for low (I) and high (II) concentrations of the analyte. Calibration standards for the calibration curves II were 20-fold diluted with citric acid solution during preparation, as described below, to avoid overloading of the MS/MS detector. The calibration curve for determination of TREO in rat brain had one uniform range. The TREO and S,S-EBDM calibration curves ranges are shown in Table 1.

Drug-free tissue homogenate supernatants were obtained by mechanical homogenization of each 1 g of the intact rat tissue with 5 mL of 0.05 M citric acid solution followed by centrifugation (4000 g for 10 min). A volume of 10 μL of the TREO and S,S-EBDM standard solutions and 10 μL of the 0.5 mM IS (codeine) solution were transferred into micro-test tubes that contained 100 μL of the drug-free rat liver, lung, muscle or brain homogenate supernatant and vortexed. To prepare the kidney homogenate supernatant calibration standards, 50 μL of the analytes standard solutions, 10 μL of the IS solution, and 50 μL of the supernatant were used. Blank and zero samples were also prepared. The contents of the micro-test