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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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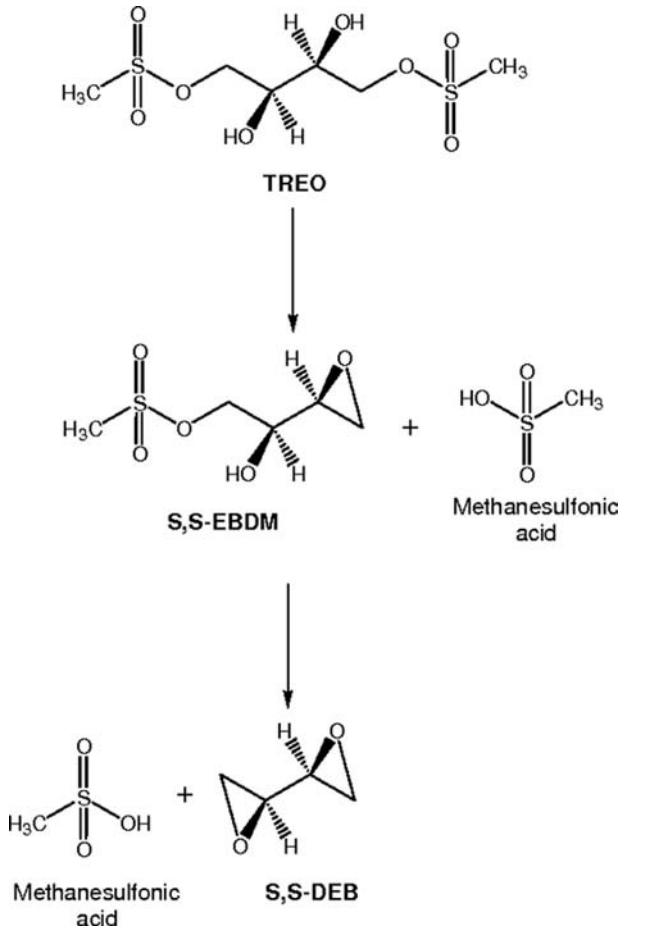


**Fig. 1.** Non-enzymatic transformation of TREO to its epoxides with alkylating activity.

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

**Table 1**
Standard solutions of TREO and S,S-EBDM.

| Standard solution no. | Nominal concentration [μM] | |
|---|---|---|
| | TREO | S,S-EBDM |
| 1 | 0.23 | 0.35 |
| 2 | 0.57 | 0.87 |
| 3 | 1.1 | 1.7 |
| 4 | 2.3 | 3.5 |
| 5 | 5.7 | 8.7 |
| 6 | 11 | 17 |
| 7 | 23 | 35 |
| 8 | 57 | 87 |
| 9 | 114 | 174 |
| 10 | 172 | 262 |
| 11 | 229 | 349 |
| 12 | 286 | 436 |
| 13 | 572 | 873 |
| 14 | 1144 | 1746 |
| 15 | 2860 | 4364 |
| 16 | 4576 | 6983 |
| 17 | 5720 | 8729 |

**Table 2**
MS/MS parameters for detection of TREO, S,S-EBDM and IS.

| Compound | Parent ion (*m*/*z*) | Daughter ion (*m*/*z*) | Fragmentor voltage [V] | Collision energy [eV] |
|---|---|---|---|---|
| TREO | 296.0 | 279.1[a] | 50 | 5 |
| | 296.0 | 183.1 | 50 | 5 |
| | 296.0 | 87.1 | 50 | 9 |
| S,S-EBDM | 200.1 | 87.1[a] | 50 | 5 |
| IS | 152.1 | 110.1[a] | 94 | 13 |
| | 152.1 | 65.1 | 94 | 33 |

[a] Transition used for quantification.

ultrasonic bath (UM-4 Unitra, Poland). A volume of 5 μL of the samples was injected into the column.

The ESI source of the MS-detector operated in a positive ionization mode. For nebulization a nitrogen stream at 40 psi (275.8 kPa) was applied. A drying gas, also nitrogen, was delivered at a flow rate of 8 L $min^{-1}$ at 300 °C. The electrospray needle voltage was 4000 V. MS/MS detection was processed in multiple reaction monitoring (MRM) mode (Table 2). The MassHunter workstation software (Agilent Technologies, USA) was used for the instrument control, data acquisition and data analysis.

### 2.4. Preparation of calibration standards and QC samples for determination of TREO and S,S-EBDM in plasma

For quantification of TREO in plasma, two calibration curves were prepared which covered low and high concentrations: calibration curve I (0.23–114 μM) and calibration curve II (114–5720 μM). The high concentration-standards for calibration curve II were more diluted during their preparation, as described below. Such a procedure was designed to avoid overloading of the MS-detector with TREO and suppression of its signal. Thus it was possible to obtain a linear plot of the calibration curves for the wide range of TREO concentrations in rat plasma.

A volume of 50 μL of the TREO and S,S-EBDM standard solutions (Nos. 1–9, 11, 13–17) was transferred into 0.2 mL micro-test tubes containing 52.5 μL of the drug-free rat plasma adjusted to pH below 5.0 (during the collection 1 mL of the drug-free rat blood was treated with 25 μL of 1 M citric acid solution). Thereafter, 10 μL of 50 μM aqueous solution of the IS was added and the contents was vortexed. The blank sample contained 60 μL of water and 52.5 μL of the drug-free rat plasma, while the zero sample consisted of 50 μL of water, 52.5 μL of the drug-free rat plasma and 10 μL of 50 μM IS solution. The samples were transferred into centrifugal filters Amicon Ultra 0.5 mL 30 K (cut-off 30 kDa) (Millipore, USA) and centrifuged at 14,000*g* at 20 °C over 20 min. For preparation of TREO calibration curve I and S,S-EBDM calibration curve, 40 μL of the obtained filtrate was transferred into 1.5-mL screw cap glass vials (Agilent Technologies, USA), diluted with 200 μL of water and vortexed. In order to prepare TREO calibration curve II, 4 μL of the obtained filtrate was diluted with 1000 μL of 250 μM citric acid solution in order to prevent an excessive increase of pH during the intensive dilution, thus prevent the artificial activation of TREO. A volume of 5 μL of the resulting solutions was injected into the HPLC-MS/MS system. Concentration of TREO and S,S-EBDM in the obtained plasma calibration standards corresponded to their concentration in the used standard solutions. Concentration of TREO in the QC samples was 0.23 μM, (LLOQ), 0.57 μM (low QC), 57 μM (medium QC) and 114 μM (high QC) for calibration curve I, and 114 μM (LLOQ), 229 μM (low QC), 2860 μM (medium QC) and 4576 μM (high QC) for calibration curve II. Concentration of S,S-EBDM in the QC samples was 0.87 μM (LLOQ), 0.87 or 1.7 μM (low QC), 87 μM (medium QC) and 174 μM (high QC). All the plasma calibration standards and the QC samples of TREO and S,S-EBDM were freshly prepared each time before the analysis.

### 2.5. Preparation of calibration standards and QC samples for determination of TREO and S,S-EBDM in the brain homogenate supernatant

A volume of 10 μL of the TREO and S,S-EBDM standard solutions (Nos. 4–5, 7–12) was transferred into 0.2 mL micro-test tubes containing 100 μL of the drug-free rat brain homogenate supernatant adjusted to pH below 5.0 (during the collection, each 1 g of the drug-free rat brain was homogenized with 5 mL of 0.05 M citric acid solution and then centrifuged in order to obtain the supernatant). Thereafter, 10 μL of 50 μM of the IS solution was added and the contents was vortexed. The blank sample contained 20 μL of water and 100 μL of the drug-free supernatant, while the zero sample consisted of 10 μL of water, 100 μL of the drug-free supernatant and 10 μL of the IS solution. The samples were then transferred into the ultrafiltration vials and centrifuged at 14,000*g* at 20 °C over 20 min. A volume of 5 μL of the resulting filtrate was injected into the HPLC-MS/MS system. Concentration of TREO and S,S-EBDM in the obtained brain homogenate supernatant calibration standards was 0.23, 0.57, 2.3, 5.7, 11, 17, 23 and 29 μM, and 0.35, 0.87, 3.5, 8.7, 17, 26, 35 and 44 μM, respectively. Concentration of TREO in the QC samples was 0.23 μM (LLOQ), 0.57 μM (low QC), 11 μM (medium QC) and 23 μM (high QC). Concentration of S,S-EBDM in the QC samples was 0.35 μM (LLOQ), 0.87 μM (low QC), 17 μM (medium QC) and 35 μM (high QC). All the brain homogenate supernatant calibration standards and the QC samples of TREO and S,S-EBDM were freshly prepared each time before the analysis.

### 2.6. Preparation of the incurred samples

The incurred samples of plasma and the brain homogenate supernatant were prepared as the corresponding calibration standards (Sections 2.4 and 2.5), except that water was added instead of the standard solution of TREO and S,S-EBDM. For determination of TREO in plasma, two aliquots were prepared from the plasma filtrate, each one corresponding to TREO calibration curve I and II.

Concentrations of the analytes in the samples were calculated from equations of the appropriate calibration curves always prepared together with the study samples as one single batch.

### 2.7. Method validation

Determination of TREO and S,S-EBDM in rat plasma and the brain homogenate supernatant by the HPLC-MS/MS method was validated according to the current guidelines of the European Medicines Agency (EMA) for bioanalytical methods [20]. It comprised selectivity, carry-over, calibration curves, within-run and between-run accuracy and precision, matrix effect, dilution integrity and stability tests.

#### 2.7.1. Selectivity

Selectivity was tested using six specimens of the drug-free rat plasma and brain homogenate supernatant obtained from the individual rats. The absence of the interfering components was accepted when the response observed at the retention time of the analytes and the IS was less than 20% of the LLOQ for the analyte and 5% for the IS.

#### 2.7.2. Carry-over

Carry-over was assessed by injecting three blank samples after the calibration standard at the upper limit of quantitation (ULOQ). A lack of carry-over was stated when the detector response observed at the retention time of the analytes and the IS was less than 20% of the LLOQ for the analyte and 5% for the IS.

#### 2.7.3. Calibration curves

Linearity of the calibration curves ($n=6$) was estimated for a peak area of the analyte to IS (acetaminophen) ratio as a function of the calibration standard concentration that provided an acceptable accuracy (within $\pm 15\%$ of the nominal value, except for $\pm 20\%$ for the LLOQ) and precision (coefficient of variation (CV) not greater than $\pm 15\%$, except for $\pm 20\%$ for the LLOQ). Mandel's fitting test was applied to confirm the linearity and Student's *t*-test was used to determine whether the *y*-intercept is statistically equal to zero.

#### 2.7.4. Accuracy and precision

Accuracy and precision were assessed on samples spiked with the known amounts of the analyte (QC samples) prepared independently from the calibration standards. Within-run accuracy and precision were determined by analyzing in a single run five samples per level at four concentration levels: the LLOQ, low QC, medium QC and high QC. For validation of the between-run accuracy and precision, the mentioned QC samples were analyzed in three runs on at least two different days.

#### 2.7.5. Dilution integrity

For evaluation of the dilution integrity, a volume of 475 μL of the drug-free rat plasma was spiked with 25 μL of the aqueous solution of 2403 μM TREO and 3666 μM S,S-EBDM so that the concentrations of the analytes in the sample were 120 and 183 μM, respectively, that is above the ULOQ which amounted to 114 μM for TREO calibration curve I and 174 μM for S,S-EBDM. A volume of 26.2 and 47.2 μL of the above plasma was then diluted with the drug-free plasma to obtain a final volume of 52.5 μL ($n=5$) and analyzed as described in Section 2.4. Dilution integrity was confirmed when the dilution process did not affect the accuracy and precision of the determinations by more than $\pm 15\%$.

#### 2.7.6. Matrix effect

In order to evaluate the influence of the co-eluting matrix components on the MS/MS detection of the analytes, the matrix effect was investigated using six lots of the drug-free plasma and brain homogenate supernatant obtained from the individual rat donors. The matrix factor (MF) was calculated for TREO, S,S-EBDM and the IS as a ratio of the peak area measured by analyzing the matrix spiked with the analytes and the IS after the ultrafiltration to the peak area in absence of the matrix. The determination was performed at the low and high level of the analytes concentration (the low and high QC samples). The IS-normalized MF was finally calculated by dividing the MF of the analyte by the MF of the IS. The matrix effect was accepted when CV of the IS-normalized MF obtained from six lots of the matrix was not greater than 15%.

#### 2.7.7. Stability tests

Stability of TREO and S,S-EBDM in the matrices was evaluated at concentrations of 0.57, 114, 5000 μM of TREO and 0.87, 174 μM of S,S-EBDM in plasma, and 0.57, 23 μM of TREO and 0.87, 35 μM of S,S-EBDM in the brain homogenate supernatant. The test samples were prepared by spiking 95 volumes of the drug-free matrix with 5 volumes of the appropriate 20-fold more concentrated standard solutions that contained 11 μM TREO and 17 μM S,S-EBDM, 458 μM TREO and 698 μM S,S-EBDM, 2288 μM TREO and 3492 μM S,S-EBDM, or 100 mM of alone TREO. The samples were analyzed by the developed HPLC-MS/MS method after 4 h of standing at room temperature (*short term stability*), after 4 months of storage at −80 °C (*long term stability*), and after 4 months of storage at −80 °C during which two freeze/thaw cycles were performed (*freeze and thaw stability*). Moreover, stability of the QC samples in the autosampler was analyzed at the following concentration levels: 0.57, 114 μM of TREO (within a range of calibration curve I), 114, 4576 μM of TREO (within a range of calibration curve I) and 0.87, 174 μM of S,S-EBDM in plasma, and 0.57, 23 μM of TREO and 0.87, 35 μM of S,S-EBDM in the brain homogenate supernatant. The QC samples were injected into the HPLC system immediately after their preparation and then after 24 h (plasma) and 16 h (brain homogenate supernatant) of standing in the autosampler at room temperature. In all the conducted tests three replicates were prepared for each concentration level. Concentration of TREO and S,S-EBDM after the storage period was calculated using the calibration curve, obtained from the freshly prepared calibration standards in the same analytical run. The analytes were considered to be stable if the deviation from the nominal concentration was within $\pm 15\%$ [20].

### 2.8. in vivo application

In order to confirm the usefulness of the method, it was applied to the analysis of plasma and brain tissue obtained from six male 34-days old CD® rats which received 500 mg (1.80 mmol) of TREO per 1 kg of the body weight. The animal experiment was performed at Laboratory of Pharmacology and Toxicology (Hamburg, Germany) upon approval by the local Ethical Committee. The rats, supplied by Charles River Laboratories (Sulzfeld, Germany), were housed in a 12-h light/dark temperature and humidity-controlled room with free access to water and food. TREO was administered as a single intravenous bolus of the 50 mg $mL^{-1}$ solution of TREO in water for injection into the tail vein (10 mL per kg). Blood was withdrawn from retrobulbar venous plexus under isoflurane anesthesia and immediately acidified by addition of 50 μL of 1 M citric acid solution per 1 mL of blood in order to avoid the artificial conversion of TREO and S,S-EBDM. Immediately after the blood sampling, the animals were sacrificed under ether anesthesia. The brain of the animals was dissected, washed in 0.9% NaCl and

divided along the longitudinal axis. One of the brain hemispheres were again washed three times with 5 mL of 0.9% NaCl, weighed and homogenized with 0.05 M citric acid solution (5 mL per 1 g of brain) in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 4000 × *g* within 10 min in order to obtain the solid particles free-supernatant. Both the acidified rat plasma and the brain homogenate supernatant samples were frozen at −80 °C and transported to the bioanalytical laboratory in dry ice at −78.5 °C within 24 h to ensure the stability of the analytes. Concentration of TREO and S,S-EBDM in the obtained incurred samples was quantified as described in Sections 2.4 and 2.5. Concentration of the analytes in the brain tissue was calculated knowing that 1 μM in the brain homogenate supernatant corresponded to 6 nmol $g^{-1}$ in the brain tissue.

## 3. Results and discussion

### 3.1. MS/MS-detection

The reported study was aimed at developing a rapid method for simultaneous determination of low concentrations of TREO and S,S-EBDM in plasma and CNS tissue for application to pharmacokinetic studies. For that purpose the HPLC-ESI-MS/MS was employed as it offers higher sensitivity and selectivity of the quantitative analysis of drugs in complex biological matrices in comparison to the other analytical techniques, especially the HPLC-RID used so far in bioanalysis of TREO. Secondly, the usage of the MS/MS-detection eliminated the need of a time-consuming and laborious derivatization, necessary for determination of S,S-EBDM by the HPLC-UV, which up to date has been the only published method regarding bioanalysis of S,S-EBDM [9]. Furthermore, due to skipping the derivatization step in the HPLC-MS/MS technique presented here, isolation of the analytes from the biological matrices could be achieved by ultrafiltration, instead of a liquid–liquid extraction required in the mentioned HPLC-UV method. Thus it became feasible to determine the biologically active protein-free fraction of S,S-EBDM instead of its total plasma concentration [9]. In the developed method acetaminophen was chosen as the IS because a certified reference standard of this compound is not expensive, readily available from the commercial suppliers and has been earlier successfully applied as the IS in the other HPLC-ESI-MS/MS methods where it provided the acceptable matrix effect during analysis of rat as well as human plasma [21,22]. Moreover, in our previous HPLC-RID method retention of acetaminophen on the reversed phase column (C18) was comparable to TREO and its epoxy-transformers [14]. Also similarly to TREO and S,S-EBDM, acetaminophen demonstrates relatively good solubility in water (14 mg/ml) and low plasma protein binding (about 24%) [9,14,23,24]. Consequently, it was supposed to mimic a behavior of TREO and S,S-EBDM in the ultrafiltration process that was applied in the developed HPLC-MS/MS-method to isolate the analytes from rat plasma and the brain homogenate supernatant.

The applied analytical procedure had to take into account the distinct chemical properties of TREO and S,S-EBDM to ensure their stability. TREO undergoes the non-enzymatic conversion to intermediate S,S-EBDM and then to S,S-DEB via intramolecular nucleophilic substitution. Velocity of those consecutive reactions increases with pH and becomes negligible when pH is decreased to at least 5 [4,9]. On the other hand, aliphatic epoxides, such as S,S-EBDM, are the most stable at neutral pH because both hydronium and hydroxide ions catalyze the oxirane ring opening which

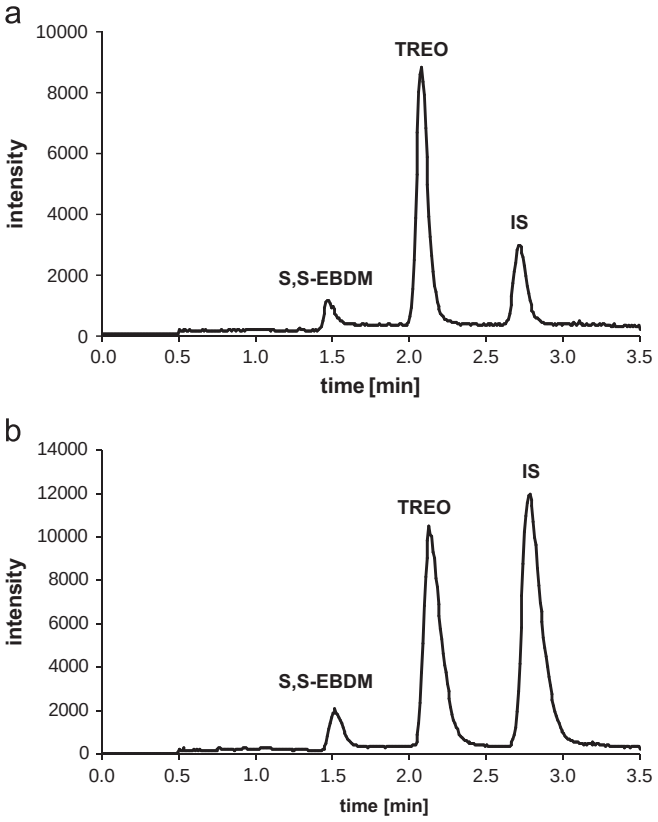


**Fig. 3.** Representative total ion current chromatograms obtained during the analysis of the high QC samples of TREO and S,S-EBDM in rat plasma (a) and the brain homogenate supernatant (b).

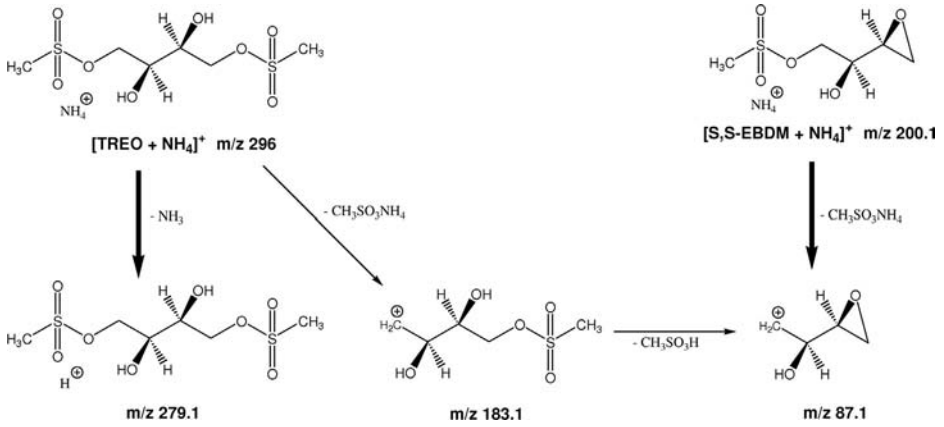


**Fig. 2.** Scheme of the proposed collision-induced fragmentation of $[M+NH_4]^+$ adducts of TREO and S,S-EBDM. Bold arrows show the transitions used for quantitation.

is called a specific catalysis of the epoxide hydrolysis. Additionally, under acidic conditions the rate of hydrolysis of the epoxides depends significantly on the concentration of the acid form of the buffer – it is a general acid catalysis of the epoxide hydrolysis [25]. Hence, in order to avoid the artificial pH-dependent epoxy-transformation of TREO and S,S-EBDM during the chromatographic run and also prevent the specific acid-catalyzed hydrolysis of the oxirane ring in S,S-EBDM, the formate buffer at pH 4.0 was applied as the main component of the mobile phase in the presented HPLC-MS/MS method [4,9]. Moreover, to avoid the general catalysis of S,S-EBDM hydrolysis, the low concentration of 0.01 M of the formate buffer was applied.

Considering the obligatory acidic character of the mobile phase as well as the presence of the electronegative oxygen-bearing groups in TREO, S,S-EBDM and acetaminophen (IS), ionization of the compounds was performed using positive electrospray mode. Optimization of the MS/MS detection settings showed that among the tested $Na^+$, $K^+$, $NH_4^+$ and $H^+$-deriving ionization products, the adducts of TREO and S,S-EBDM with $NH_4^+$ and the IS with $H^+$ were formed predominantly, therefore they were used as the parent ions for quantification. Here it is worth to note that S,S-DEB, a final product of TREO transformation, was also present in the tested solutions but could not be detected since it undergoes no ionization under the ESI conditions [26]. In contrary, molecules of TREO and S,S-EBDM were very susceptible to the applied positive ionization as the amount of $[M+NH_4]^+$ adducts formed was greatest already at the smallest tested fragmentor voltage of 50 V and continually decreased with the voltage increase up to 300 V. Preferential formation of the ammonium precursor ions at low cone voltages has been also observed earlier in the MS detection of busulfan which structurally differs from TREO only in a lack of two hydroxyl groups in C-2 and C-3 positions of the butane chain [27–29]. This suggest that $[M+NH_4]^+$ adducts of TREO and S,S-EBDM were formed due to electrostatic attraction between the $NH_4^+$ cations and the free electron pairs of oxygen atoms located in the methanesulfonic group, not in the hydroxyl group. The subsequent fragmentation of the produced $[M+NH_4]^+$ parent ions of TREO and S,S-EBDM also run readily as within the tested range of the collision energies of 5–45 eV, the lowest one generally provided the maximal amount of the daughter ions of which $m/z$ values are depicted in Table 2. The three product ions registered for TREO were formed in similar amounts, however 296/279.1 transition provided the best analyte tracking in terms of accuracy and precision, therefore was chosen for quantification. In opposition to TREO, fragmentation of the S,S-EBDM ammonium adduct produced only one daughter ion at $m/z$ 87.1. Among the two daughter ions of acetaminophen (IS), observed at $m/z$ 110.0 and 65.1, the former was more abundant (ratio 100:35) and, therefore, was applied to quantitative analysis. The proposed structures of the product ions of the both analytes are presented in Fig. 2. The daughter ion of TREO registered at $m/z$ 279.1 was supposed to origin from a loss of neutral $NH_3$ molecule from the parent ion $[M+NH_4]^+$. This type of cleavage is often met in

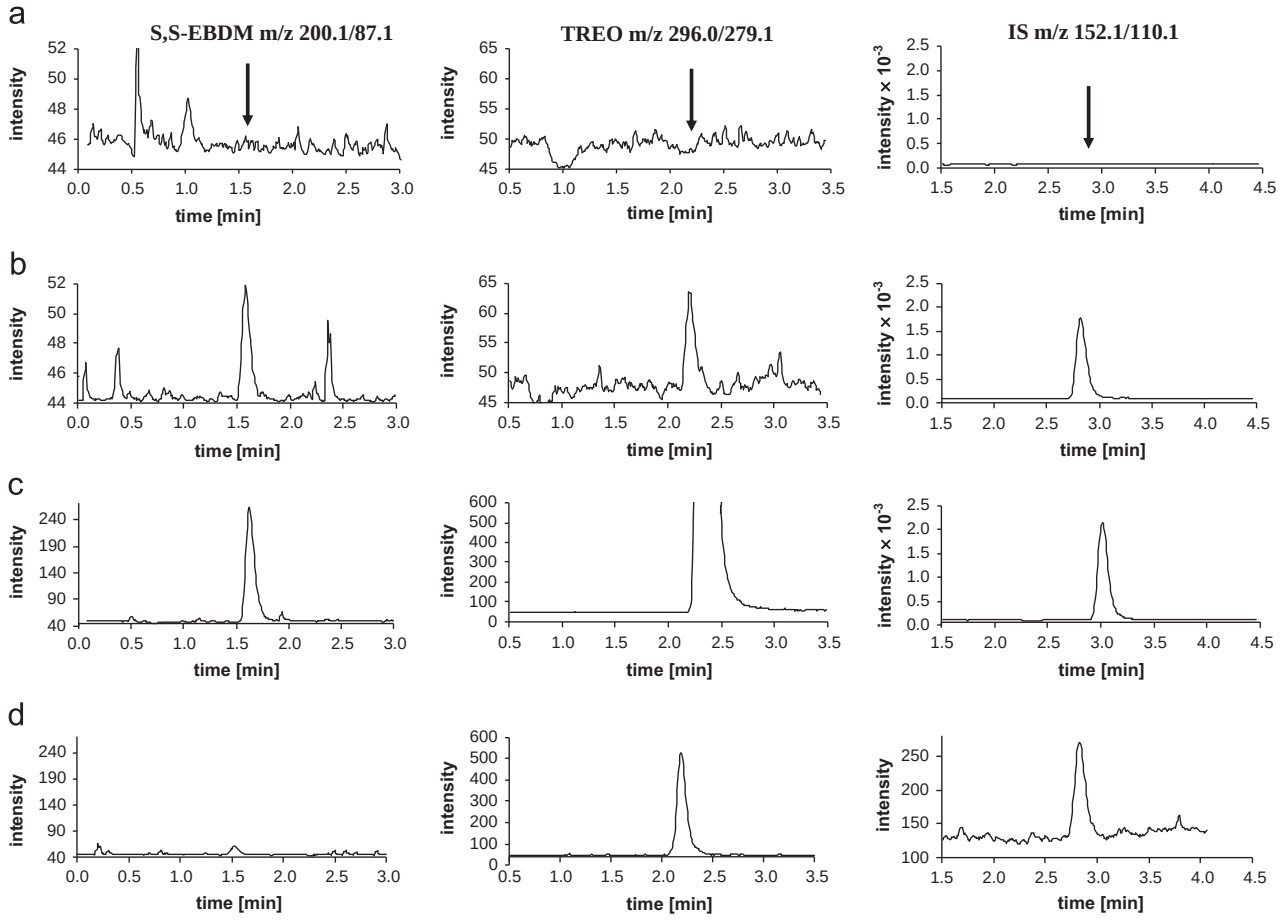


**Fig. 4.** Extracted ion current chromatograms of S,S-EBDM, TREO and the IS (acetaminophen) acquired during the analysis of plasma: (a) blank sample; (b) calibration standard at the LLOQ level, *i.e.* 0.87 μM for S,S-EBDM and 0.23 μM for TREO; (c) incurred rat plasma obtained at 1 h after the intravenous bolus of TREO 500 mg/kg, prepared according to the procedure for TREO calibration curve I (0.23–114 μM) and S,S-EBDM calibration curve (determined concentration of S,S-EBDM 39 μM); (d) incurred rat plasma obtained at 1 h after the intravenous bolus of TREO 500 mg/kg, prepared according to the procedure for TREO calibration curve II (114–5720 μM) (determined concentration of TREO 826 μM).

fragmentation of the ammonium adducts, however, no analogical product was found for S,S-EBDM [30,31]. The only transition found for S,S-EBDM, namely *m*/*z* 200.1/87.1, as well as transition *m*/*z* 296/183.1 found for TREO corresponded to a loss of 113 Da from the appropriate parent ions. The same mass reduction was observed earlier during fragmentation of the ESI-induced ammonium adduct of busulfan to the daughter ion [27]. The above collision-induced fragmentation path, common for TREO, S,S-EBDM and busulfan, most likely results from the cleavage of ammonium methanesulfonate from their molecules, occurring due to heterolytic fission of the ester C–O bond. In case of TREO the produced daughter ion at *m*/*z* 183.1 presumably underwent further fragmentation by a loss of the methanesulfonic acid molecule, which led to creation of the third daughter ion of TREO, registered at *m*/*z* 87.1 (Fig. 2).

### 3.2. Validation results

#### 3.2.1. Selectivity, carry-over

The applied chromatographic conditions provided a complete resolution of TREO, S,S-EBDM and the IS during their analysis in plasma as well as the brain homogenate supernatant (Fig. 3). The result is similar to that obtained in the HPLC-RID method developed previously for determination of TREO and its epoxy-transformers in the pure phosphate buffer [14]. However, in the present study significant reduction of the chromatographic run time was achieved, that is from 8 to about 3 min, owing to the usage of the shorter but more efficient HPLC column – Zorbax Eclipse Plus (100 × 2.1 mm) packed with 3.5 μm particles instead of Hypersil ODS (150 × 4.6 mm) packed with 5 μm particles. Noteworthy, the obtained run time was also much shorter when

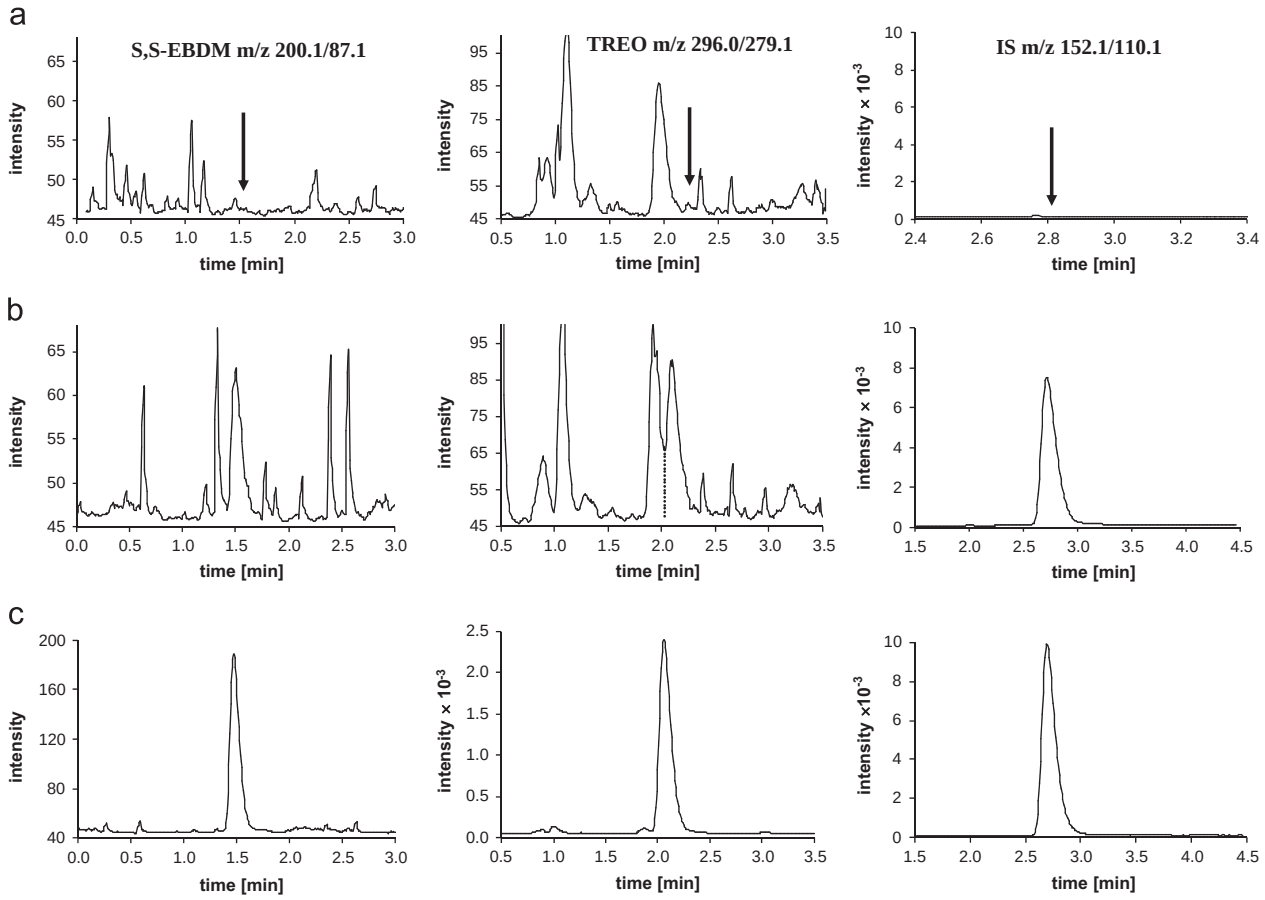


**Fig. 5.** Extracted ion current chromatograms of S,S-EBDM, TREO and the IS (acetaminophen) acquired during the analysis of the brain homogenate supernatant: (a) blank sample; (b) calibration standard at the LLOQ level, *i.e.* 0.35 μM for S,S-EBDM and 0.23 μM for TREO; (c) incurred brain homogenate supernatant sample obtained at 1 h after the intravenous bolus of TREO 500 mg/kg (determined concentrations: TREO 13.0 μM; S,S-EBDM 2.7 μM).

**Table 3**
Calibration curves for determination of TREO and S,S-EBDM in rat plasma and brain homogenate supernatant.

| Matrix | Analyte | Range (μM) | Model preferred in Mandel's test: linear/quadratic | Correlation coefficients for curves $y=ax$ | Equation of a mean linear calibration curve $y=ax$ |
|---|---|---|---|---|---|
| Plasma | TREO | 0.23–114 (I) | 4/2 | ≥ 0.9992 | $P_{TREO}/P_{IS}=1.416\times10^{-2}\times C_{TREO}$ |
| | | 114–5720 (II) | 6/0 | ≥ 0.9992 | $P_{TREO}/P_{IS}=1.187\times10^{-2}\times C_{TREO}$ |
| | S,S-EBDM | 0.87–174 | 3/3 | ≥ 0.9995 | $P_{S,S\text{-}EBDM}/P_{IS}=2.272\times10^{-3}\times C_{S,S\text{-}EBDM}$ |
| Brain homogenate supernatant | TREO | 0.23–28 | 5/1 | ≥ 0.9988 | $P_{TREO}/P_{IS}=2.835\times10^{-2}\times C_{TREO}$ |
| | S,S-EBDM | 0.35–44 | 4/2 | ≥ 0.9982 | $P_{S,S\text{-}EBDM}/P_{IS}=4.348\times10^{-3}\times C_{S,S\text{-}EBDM}$ |

$n=6$ curves for each analyte in each matrix.

compared to 30 min required for the chromatographic analysis of the derivatized products of S,S-EBDM by the mentioned HPLC-UV method [9]. Quantification of TREO and S,S-EBDM in plasma by the developed HPLC-MS/MS technique was highly selective as no interfering peaks of the endogenous compounds were observed within the retention times of the analytes and the IS (Fig. 4). Some additional peaks, which originated from the matrix components, were present on the chromatograms of the brain homogenate supernatant samples (Fig. 5). However, they did not disturb the accurate and precise determination of the analytes within the whole range of the calibration curve. In addition, no measurable carry-over of TREO, S,S-EBDM and the IS was found in the blank rat plasma as well as the blank brain homogenate supernatant samples after injection of the highest calibration standards.

### 3.2.2. Linearity, LLOQ

Parameters of the calibration curves for determination of TREO and S,S-EBDM in rat plasma and the brain homogenate supernatant are presented in Table 3. Mandel's fitting test proved that 16 out of the 24 prepared curves were better described by a linear model than a quadratic one. Moreover, for all the calibration curves the linear model $y=ax$ ($y$-intercept values were not statistically different from zero in $t$-Student's test) provided good fit of the data ($r \geq 0.998$) and acceptable accuracy of determinations as the back calculated concentrations of the calibration standards were within $\pm 20\%$ of the nominal value for the LLOQ and within $\pm 15\%$ for the other concentrations. For that reason, and following the EMA guidelines stating that 'a relationship which can simply and adequately describe the response of the instrument with regard to the concentration of analyte should be applied', the linear calibration curves $y=ax$ were finally established for determination of TREO and S,S-EBDM in the both studied matrices [20].

In the developed HPLC-MS/MS method values of the LLOQ corresponded to the lowest concentrations of the analytes on the calibration curves (Table 3). The LLOQ for TREO in plasma and the brain homogenate supernatant, equal to 0.23 μM, was much lower than 4–180 μM, obtained earlier during the analysis of TREO in human plasma and urine by the HPLC-RID method [6,13]. In turn the LLOQ values for S,S-EBDM proved to be lower than 2.5 μM reported in the HPLC-UV method for determination of this epoxide in human plasma after derivatization with 3-nitrobenzenesulfonic acid [9].

**Table 4**
Precision and accuracy for determination of QC samples of TREO and S,S-EBDM in rat plasma and brain homogenate supernatant.

| QC level ($n=5$) | Precision (CV%) | | Accuracy (%) | |
|---|---|---|---|---|
| | Within-run | Between-run | Within-run | Between-run |
| *Determination of TREO in plasma (range of calibration curve I)* | | | | |
| LLOQ 0.23 μM | 2.2 | 7.2 | 102.2 | 103.5 |
| Low QC 0.57 μM | 5.8 | 12.1 | 105.8 | 97.8 |
| Mid QC 57 μM | 2.2 | 4.7 | 103.7 | 99.2 |
| High QC 114 μM | 1.0 | 6.2 | 94.8 | 94.8 |
| *Determination of TREO in plasma (range of calibration curve II)* | | | | |
| LLOQ 114 μM | 1.9 | 1.7 | 102.3 | 105.3 |
| Low QC 229 μM | 2.2 | 6.4 | 102.4 | 107.0 |
| Mid QC 2860 μM | 5.1 | 6.2 | 89.4 | 96.7 |
| High QC 4576 μM | 1.8 | 12.0 | 90.3 | 98.0 |
| *Determination of S,S-EBDM in plasma* | | | | |
| LLOQ 0.87 μM | 12.9 | 13.6 | 92.8 | 99.0 |
| Low QC 1.7 μM | 3.4 | 5.9 | 102.7 | 106.3 |
| Mid QC 87 μM | 2.6 | 1.8 | 99.6 | 100.3 |
| High QC 174 μM | 2.6 | 3.2 | 91.1 | 96.0 |
| *Determination of TREO in brain homogenate supernatant* | | | | |
| LLOQ 0.23 μM | 3.2 | 6.6 | 104.3 | 115.1 |
| Low QC 0.57 μM | 2.9 | 7.6 | 97.0 | 108.1 |
| Mid QC 11 μM | 2.9 | 10.2 | 99.2 | 97.5 |
| High QC 23 μM | 1.9 | 2.5 | 92.7 | 97.8 |
| *Determination of S,S-EBDM in brain homogenate supernatant* | | | | |
| LLOQ 0.35 μM | 5.6 | 12.5 | 95.6 | 104.7 |
| Low QC 0.87 μM | 6.5 | 7.4 | 101.1 | 114.0 |
| Mid QC 17 μM | 2.8 | 10.2 | 95.9 | 97.5 |
| High QC 35 μM | 2.0 | 9.7 | 88.6 | 99.3 |

### 3.2.3. Accuracy and precision

The HPLC-MS/MS method provided high within-run and between-run precision as well as accuracy of determination of TREO and S,S-EBDM in rat plasma and the brain homogenate supernatant. At the four QC samples concentration levels that covered the whole range of the linear calibration curves, including the LLOQ, the values of CV were $\leq 13.6\%$ and accuracy ranged from 88.6% to 115.1% (Table 4).

### 3.2.4. Matrix effect

Since an ESI-MS/MS detection is inherently affected by the matrix components co-eluted with the analytes, the matrix effect was investigated for plasma and the brain homogenate supernatant obtained from the individual rat donors. The values of MF, expressing the detector response in presence of the matrix in relation to the neat solution, varied from 0.66 to 1.37 for TREO, 0.72–1.21 for S,S-EBDM, and 0.71–1.08 for IS (Table 5), reflecting either moderate enhancement or suppression of the signal. The calculated values of the IS-normalized MF were always close to one, which indicates that the matrices had the similar influence on ionization and further detection of the either analyte and the IS. Moreover, the results were repeatable within the six different studied matrix specimens ($CV < 15\%$), which confirms that the possible matrix effect did not disturb precision of the quantitative analysis of TREO and S,S-EBDM.

### 3.2.5. Dilution integrity

In order to evaluate the dilution integrity, plasma QC sample at concentration above the ULOQ was diluted by 10 and 100% with the blank matrix. The resulting samples contained nominally 108 μM TREO and 165 μM S,S-EBDM (dilution factor 1.1), and 60 μM TREO and 92 μM S,S-EBDM (dilution factor 2). In the

**Table 5**
Matrix effect observed during ESI-MS/MS-detection of TREO and S,S-EBDM in rat plasma and brain homogenate supernatant.

| Nominal concentration of the analyte (μM) | MF range | | IS-normalized MF ($n=6$) | | |
|---|---|---|---|---|---|
| | Analyte | IS | Mean ± SD | Precision (CV%) | |
| *TREO in plasma (range of calibration curve I)* | | | | | |
| 0.57 | 0.86–0.99 | 0.81–0.92 | 1.07 ± 0.02 | 2.0 | 13.3 |
| 114 | 1.08–1.37 | 0.87–0.95 | 1.3 ± 0.1 | 8.6 | |
| *TREO in plasma (range of calibration curve II)* | | | | | |
| 114 | 0.94–1.00 | 0.83–0.92 | 1.12 ± 0.02 | 2.0 | 5.5 |
| 4576 | 0.98–1.07 | 0.90–1.08 | 1.03 ± 0.05 | 5.1 | |
| *S,S-EBDM in plasma* | | | | | |
| 0.87 | 0.82–1.02 | 0.81–0.92 | 1.0 ± 0.1 | 10.2 | 11.4 |
| 174 | 1.09–1.21 | 0.87–0.95 | 1.2 ± 0.1 | 9.4 | |
| *TREO in brain homogenate supernatant* | | | | | |
| 0.57 | 0.66–1.03 | 0.72–1.00 | 1.0 ± 0.1 | 5.7 | 11.1 |
| 23 | 0.84–1.15 | 0.71–0.96 | 1.22 ± 0.04 | 3.5 | |
| *S,S-EBDM in brain homogenate supernatant* | | | | | |
| 0.87 | 0.72–1.19 | 0.72–1.00 | 1.1 ± 0.1 | 6.2 | 5.5 |
| 35 | 0.77–0.98 | 0.71–0.96 | 1.06 ± 0.05 | 4.4 | |

**Table 6**
Stability of TREO and S,S-EBDM in plasma and brain homogenate supernatant samples.

| Matrix | Plasma | | | | | Brain homogenate supernatant | | | |
|---|---|---|---|---|---|---|---|---|---|
| Analyte | TREO | | | S,S-EBDM | | TREO | | S,S-EBDM | |
| Nominal concentration (μM) | 0.57 | 114 | 5000 | 0.87 | 175 | 0.57 | 29 | 0.87 | 44 |
| **Short term stability (4 h at room temperature)** | | | | | | | | | |
| Mean assayed value (μM) | 0.57 | 101 | 5060 | 0.85 | 175 | 0.61 | 29 | 0.94 | 43 |
| Accuracy (%) | 99.8 | 88.6 | 101.2 | 97.9 | 100.5 | 106.3 | 101.6 | 108.2 | 97.6 |
| **Long term stability (4 months at −80 °C)** | | | | | | | | | |
| Mean assayed value (μM) | 0.64 | 102 | 5100 | 0.85 | 154 | 0.60 | 28 | 0.88 | 41 |
| Accuracy (%) | 113.2 | 88.8 | 102.0 | 97.9 | 88.2 | 105.8 | 99.5 | 100.6 | 93.7 |
| **Freeze and thaw stability (2 cycles at −80 °C/room temperature)** | | | | | | | | | |
| Mean assayed value (μM) | 0.52 | 103 | 4970 | 0.80 | 159 | 0.63 | 28 | 0.86 | 43 |
| Accuracy (%) | 90.5 | 90.3 | 99.4 | 92.4 | 91.1 | 109.9 | 99.4 | 98.7 | 99.4 |
| **Autosampler stability (24 or 16 h at room temperature)** | | | | | | | | | |
| Mean assayed value (μM) | 0.60 | 100, 120[a] | 4677[b] | 0.92 | 155 | 0.51 | 27 | 0.94 | 47 |
| Accuracy (%) | 104.5 | 87.3, 104.8[a] | 102.2[b] | 105.2 | 88.8 | 89.4 | 94.3 | 108.5 | 108.6 |

$n=3$ replicates for each concentration of the analytes in each matrix.

[a] The former and the latter values are given for the samples corresponding to TREO calibration curve I (0.57–114 μM) and TREO calibration curve II (114–5720 μM), respectively.

[b] The nominal concentration of TREO in the sample was 4576 μM instead of 5000 μM.

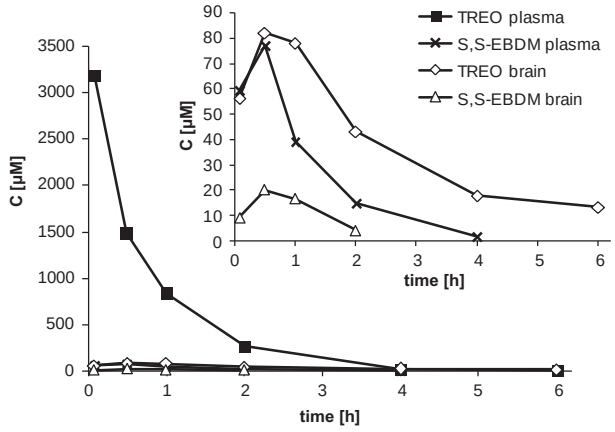


**Fig. 6.** Concentrations of TREO and S,S-EBDM in plasma and brain tissue *versus* time following intravenous bolus of 500 mg TREO per kg of the body weight to six male rats. The inset in graph illustrates the changes in concentration of S,S-EBDM in plasma and in concentrations of TREO as well as S,S-EBDM in brain tissue. Each concentration point corresponds to one rat.

prepared plasma replicates ($n=5$) the analytes were determined with accuracy 91.9–95.5% and precision (CV) 1.0–1.9%. Thus it was proved that the developed HPLC-MS/MS method can be applied to analyze plasma samples diluted due to the analytes concentration exceeding the ULOQ or due to the insufficient volume of the collected rat plasma.

### 3.2.6. Stability tests

It was demonstrated that the parent drug and S,S-EBDM were stable in each conducted stability test as their concentrations did not change by more than ±13.2% from the nominal values (Table 6). Namely, accuracy of TREO determinations fell into a range of 88.6–113.2% for plasma and 99.4–109.9% for the brain homogenate supernatant. Moreover, TREO demonstrated post-preparative stability in the both ultrafiltered matrices (cut-off 30 kDa) standing over 16–24 h in the autosampler at room temperature. These data are consistent with the previous results concerning stability of TREO in human plasma as well as urine, the human plasma ultrafiltrate and 0.073 M phosphate buffer [6,13,14]. The similar results were obtained for S,S-EBDM since in the short term, long term and freeze and thaw stability test it was determined in plasma and the brain homogenate supernatant with accuracy of 88.2–100.5% and 93.7–108.2%, respectively, and with accuracy of 88.8–108.6 in the autosampler stability test. The long term stability of S,S-EBDM in the studied matrices stored at −80 °C within 4 months is in opposition to the reported instability of this compound in 0.073 M phosphate buffer at −25 °C within 135 days [14]. This difference indicates that only the lower temperature of −80 °C was sufficient to prevent opening of the relatively reactive epoxide ring within the tested storage period.

## 3.3. In vivo studies

The validated HPLC-MS/MS method was applied to simultaneous determination of TREO and S,S-EBDM in the incurred plasma and brain samples obtained from six adult rats which received the intravenous bolus of TREO at a dose of 500 mg (1.80 mmol) per kg of the body weight. In comparison to the parent drug, concentration of S,S-EBDM in plasma was about 40-fold lower. Noteworthy, both TREO and S,S-EBDM were found in the brain tissue, though at concentrations lower than in plasma (Fig. 6). The obtained results constitute the first data on analysis of TREO and S,S-EBDM in CNS tissues and prove that the investigated species are capable of penetrating across blood–brain barrier. The values of $C_{max}$ of the compounds in the brain tissue, 82.0 and 20.0 nmol $g^{-1}$, respectively, were noted at 0.5 h after administration of the parent drug. A ratio of $C_{max}$ of TREO in plasma to the brain tissue amounted to 38.8, whereas the same parameter calculated for S,S-EBDM was 10-fold lower, namely 3.8. This indicates that S,S-EBDM demonstrates better penetration to brain than TREO, which may arise from its higher lipophilicity [32]. Obviously, penetration of anticancer agents into CNS provides their desirable cytotoxic activity against primary or metastatic brain tumors as well as causes possible undesirable adverse effects, *e.g.* seizures. Therefore, in our opinion, larger *in vivo* studies concerning penetration of TREO and its biologically active epoxy-transformers across blood–brain barrier are strongly warranted from clinical point of view.

## 4. Conclusion

The presented HPLC-MS/MS method is the first analytical technique for determination of protein-free TREO and S,S-EBDM in brain tissue and plasma at concentrations below 1 μM. It provides the convenient direct assay of TREO and S,S-EBDM in one analytical run within about 3 min. The method fulfils the validation requirements of the EMA for quantitative analysis of drugs and their metabolites in biological samples. Owing to employment of the selective and sensitive mass spectrometry detection it enabled determination of TREO and S,S-EBDM in the incurred plasma and brain samples obtained from the rats treated with the intravenous bolus of TREO.

## Conflict of interests

The studies were financially supported by medac GmbH. The sponsor in person of Dr. Sonja Bohm and Dr. Joachim Baumgart was involved in designing of the *in vivo* experiments and sampling methodology carried out by Laboratory of Pharmacology and Toxicology. Medac GmbH has not been involved in any stage of the bioanalytical studies and the manuscript preparation.

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