PHARMACOKINETICS AND METABOLISM OF A NEW POTENT ANTIEPILEPTIC DRUG, 2,2,3,3-TETRAMETHYLCYCLOPROPANECARBONYLUREA, IN RATS

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PHARMACOKINETICS AND METABOLISM OF TMCU IN RATS

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ABBREVIATIONS: TMCU, 2,2,3,3-tetramethylcyclopropanecarbonylurea;
OH-TMCU, trans-2-hydroxymethyl-2,3,3-trimethylcyclopropanecarbonylurea; UGT, uridine diphosphate glucuronisyltransferase; MES, maximal electroshock-induced seizures test; scMet, pentylenetetrazol-induced seizures test; GC/MS, gas chromatography-mass spectrometry; LC/MS, liquid chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; TLC, thin layer chromatography; COSY, correlated spectroscopy; ESI, electrospray ionization; F,
absolute bioavailability; $V_z$, volume of distribution based on linear terminal slope; $V_z/F$, $V_z$ normalized to F and calculated following extravascular dosing; $V_{ss}$, volume of distribution at steady state; AUC, area under plasma drug concentration-time curve; $AUC_m$, area under plasma metabolite concentration-time curve; AUMC, area under the first moment curve; $C_{max}$, peak plasma drug concentration; $f_e$, fraction of the systemically available drug excreted unchanged in the urine; $A_m$, cumulative amount of the metabolite excreted in the urine; $f_u$, fraction unbound in plasma.
Abstract:
The pharmacokinetics and metabolism of 2,2,3,3-tetramethyle cyclopropanecarbonylurea (TMCU), a potent anticonvulsant compound, were studied in male Sprague-Dawley rats following intravenous (i.v., 5 mg/kg), oral (20 mg/kg) and intraperitoneal (i.p., 20 mg/kg) administrations. Urine samples were analyzed by GC/MS and LC/MS. Plasma samples were analyzed by GC/MS. TMCU absolute bioavailability was 83% and 90% following oral and i.p. dosing, respectively. Following i.p. administration, the peak plasma concentration (C\text{max}) obtained 45 min after dosing was 15.4 mg/L. Following oral dosing C\text{max} was 6.5 mg/L and it was reached after 4 h. The disposition kinetics of TMCU in rats was adequately described by one-compartment open body model. TMCU is well distributed into the extravascular tissues with volume of distribution (V\text{ss}) of 0.87 L/kg and undergoes extensive metabolism. Only a small fraction of TMCU excreted unmetabolized in the urine (6.3 ± 0.8%). Trans-2-hydroxymethyl-2,3,3-trimethyle cyclopropanecarbonylurea (OH-TMCU) was a predominant metabolite of TMCU. Its structure was established by NMR and X-ray crystallography. Following i.p. administration of 5 and 20 mg/kg TMCU, the drug was excreted in the urine as OH-TMCU at an extent of  28.3 ± 2.6% and 42.1 ± 3.8%, respectively. A portion of OH-TMCU was excreted in the urine as TMCU sulfate and TMCU glucuronide.
Epilepsy is one of the most common neurological disorders, affecting about 1% of the world's population and characterized by recurrent seizure attacks (McNamara, 2001). Valproic acid (VPA, Fig. 1) is one of the most prescribed antiepileptic drugs (AEDs), which is also effective in migraine prophylaxis and treatment of bipolar disorders (McNamara, 2001; Bourgeois, 2002; Silberstein, 2002; Swann, 2002). The clinical use of VPA is restricted by two rare, but potentially life-threatening side effects, hepatotoxicity and teratogenicity, that preclude its utilization in children and women of childbearing age. (Konig et al., 1994; Konig et al, 1999; Kaneko et al, 1999). While VPA-induced hepatotoxicity is associated with the formation of metabolites with a terminal double bond like 4-ene-VPA and 2,4-diene-VPA (Fig. 1) (Sussman and McLean, 1979; Zimmerman and Ishak, 1982; Rettie et al., 1987; Baillie, 1992; Sadeque et al., 1997), VPA-induced teratogenicity is caused by the parent compound itself (Nau, 1986, Nau et al., 1991). In anticonvulsant animal models, VPA has a low antiepileptic potency compared to other major AEDs (White et al., 2002). In addition, VPA has pharmacokinetic (PK) drawbacks such as enzyme inhibition and involvement in drug interactions (Levy et al., 2002; Bowdle et al., 1979; Sackellares et al., 1981; Wagner et al., 1991; Robbins et al., 1990; Kanner and Frey, 2000; Bruni et al., 1979; Bruni et al., 1980; Hoffman et al., 1981). Due to the above-mentioned shortcomings of VPA and since about 30% of the epileptic patients are not seizure-free under existing AEDs therapy, there is a substantial need for the development of better and safer AEDs.

2,2,3,3-Tetramethylcyclopropanecarboxylic acid (TMCA, Fig. 1) is a cyclic analogue of VPA that possesses only a weak anticonvulsant activity (unpublished data). The rationale behind the synthesis of this compound was to prevent formation of hepatotoxic metabolites with a terminal double bond by creation a quaternary carbon
at β-positions to the carbonyl group. 2,2,3,3-Tetramethylcyclopropanecarbonylurea (TMCU, Fig. 1) is a urea derivative of TMCA (Sobol et al., 2004) possessing a very potent anticonvulsant activity in the animal (mice and rats) models of epilepsy such as maximal electroschock-induced seizures test (MES) and pentylenetetrazol-induced seizures test (scMet) (White et al., 2002; Sobol et al., 2004). The development of new compounds that display potent anticonvulsant activity and are less toxic than the existing AEDs is a major therapeutic goal. TMCU was 17 times more potent than VPA in the rat-MES test and its protective index (PI), presented by the ratio of toxic dose (TD$_{50}$) to the effective dose (ED$_{50}$) was 18.5. This PI value is 12 times larger than that of VPA, suggesting that TMCU may have a wider safety margin (White et al., 2002; Sobol et al., 2004). The high anticonvulsant potency and large protective index make TMCU a promising candidate for development as a novel antiepileptic drug.

The aim of the present study was to evaluate the PK and metabolism of TMCU following its intravenous (i.v.), oral and intraperitoneal (i.p.) administration to rats.
Materials and Methods

Chemicals and Reagents. All the solvents were of analytical grade and were supplied by J. T. Baker, The Netherlands. Methylcellulose, iodomethane, trimethylchlorosilane and C18 sorbent (50-63 µm particle size, 60 Å pore diameter and 500 m²/g surface area) were purchased from Sigma-Aldrich, USA. Silica-gel (Kieselgel 60, 0.2-0.5 mm) was supplied by Merck, Germany. TMCU was synthesized according to the previously reported procedure (Sobol et al., 2004). Valproyl glycinamide was kindly provided by Teva Pharmaceuticals, Israel. Double distilled water was used throughout the study. Levetiracetam was synthesized according to the previously reported procedure (Isoherranen et al., 2001).

Animals and Collection of Blood Samples and Excreta. Male Sprague-Dawley rats weighing 300 ± 24 g were used throughout the study. The animals were maintained at 22°C with a 12 h light/dark cycle. Prior to each experiment the animals were allowed to acclimatize for at least two days in communal cages with standard rat chow and water provided ad libitum. For urine collection animals were kept in metabolic cages. In all the experiments animals were fasted 12 h before and 4 h after the drug administration. Urine was collected in 50 ml plastic tubes and stored at -20 °C until analyzed. In the PK studies three rats were sacrificed at each time point and blood samples were withdrawn by cardiac puncture under deep isoflurane anesthesia. Blood samples were collected at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 15 and 20 h after dosing. The heparinized test tubes with collected blood were immediately centrifuged at 3000g for 10 min and plasma was separated and stored at -20°C until analyzed. In each PK experiment three rats kept in metabolic cages for urine collection over 24 h period. The animal use in the PK and metabolism studies was approved by the Institutional Animal Care and Use Committee.
**Drug Administration.** In the PK studies TMCU dissolved in 96% ethanol was administered intravenously (i.v., 5 mg/kg through the tail vein), orally (20 mg/kg) and intraperitoneally (i.p., 20 mg/kg). 250 µl of ethanol was the maximal injected volume. No behavioral changes were observed in rats. For initial identification of metabolites in the urine, three rats were dosed i.p. with 1 ml of TMCU suspension (40 mg/ml made in 0.5% methylcellulose in H₂O). For elucidation of metabolic profile three rats were dosed with 5 mg/kg TMCU i.p. and three rats with 20 mg/kg TMCU i.p. (1.5 mg/ml and 6 mg/ml TMCU suspension in 0.5% methylcellulose in H₂O, respectively).

**Isolation and Identification of Metabolites in Urine.**

Urine samples were chromatographed initially on C-18 SPE cartridges (1 g, Extra-Sep C18, Merck, Germany). Three cartridges were conditioned with 3 ml of methanol followed by 3 ml of water. Urine (2 ml) was loaded onto each cartridge and eluted under gravity. The cartridges were subsequently eluted with 3 ml of H₂O following by 2 ml of methanol. The methanol fraction was analyzed by GC/MS and LC/MS for the presence of TMCU metabolites.

**Isolation of the Major Metabolite of TMCU.** For isolation of the major metabolite of TMCU from the urine, seven rats were dosed i.p. with 1 ml of TMCU suspension (40 mg/ml in 0.5% methylcellulose in H₂O). Urine collected for 24 h was lyophilized, the residue was redissolved in 10 ml of H₂O and applied to the C-18 column (50 g, 35 cm * 2.5 cm), previously conditioned with 50 ml of methanol and 50 ml of water. The column was subsequently eluted with 200 ml of H₂O following by 50 ml aliquots of varying percentage of methanol in H₂O (20, 30, 40, 60, 80 and 100% methanol). The eluents were lyophilized and the residues were redissolved in 5 ml of methanol, of which 1 µl was injected into GC/MS. The 40% methanol fraction was lyophilized and the residue, redissolved in small volume of methanol, was rechromatographed on
silica-gel column (50 g, 35 cm × 2.5 cm). The metabolite was eluted using 8% methanol in dichloromethane. The eluted aliquots were analyzed by TLC (precoated silica gel on aluminum sheets, Kieselgel 60 F₂₅₄, Merck, Germany) using dichloromethane/methanol(10%) as a developing solvent. The fractions containing the metabolite were pooled, dried and crystallized using chloroform/methanol mixture (3:1). Large white crystals, melting point 120-121°C, were obtained. The purity of the metabolite was established by TLC, GC/MS and LC/MS. NMR analysis and X-ray crystallography of the crystals were performed for elucidation of its chemical structure.

**NMR Analysis.** NMR data of the metabolite were generated on a Varian Inova 500 MHz spectrometer using a broadband 5mm probehead and standard pulse sequences. The data were processed by the VNMR software or Mestrec using a sinebell apodization in both dimensions for the absolute value COSY experiment and a cosine square apodization in both dimensions for the phase sensitive spectra. Linear prediction in the second dimension was also applied. The spectra were measured in DMSO at 30°C and were referenced to the solvent.

**X-ray Crystallography of TMCU Major Metabolite.** A single crystal was coated with vaseline, attached to a glass fiber, and transferred to a Bruker SMART APEX CCD X-ray diffractometer system controlled by a pentium-based PC running the SMART software package (SMART-NT V5.6, BRUKER AXS GMBH, D-76181 Karlsruhe, Germany, 2002). The crystal was mounted on the three-circle goniometer with χ fixed at +54.76° and was rapidly cooled to -73 °C with a Bruker KRYOFLEX nitrogen cryostat. The diffracted graphite-monochromated Mo Kα radiation (λ=0.71073 Å) was detected on a phosphor screen held at a distance of 6.0 cm from the crystal operating at -44 °C. A detector array of 512 X 512 pixels, with a pixel size of approximately 120
µm, was employed for data collection. The detector centroid and crystal-to-detector
distance were calibrated from a least-squares analysis of the unit cell parameters of a
carefully centered YLID reference crystal.

After the crystal of the metabolite had been carefully optically centered within the X-
ray beam, a series of 30 data frames measured at 0.3° increments of ω were collected
with three different 2θ and ϕ values to assess the overall crystal quality and to calculate
a preliminary unit cell. In order to correct for high-energy background events in the
images, data frames were collected as the sum of two 5 sec exposures and
noncorrelating events were eliminated. A correction for the background detector current
was also applied to the frames, and a small offset was added to all of the individual
pixel values to prevent any statistical bias induced by truncating negative values to
zero. The measured intensities of individual reflections were plotted at 0.3° intervals of
ω, and the average peak width at baseline was less than 1.5°. A total of 49 reflections
with I > 20σ(I) were selected from the data frames and utilized to calculate a
preliminary unit cell. For the collection of the intensity data, the detector was
positioned at a 2θ value of -28° and the intensity images were measured at 0.3°
intervals of ω for duration of 20 sec each. The data frames were collected in four
distinct shells which, when combined, measured more than 1.3 hemispheres of intensity
data with a maximum 2θ of 52°. For the first shell, the crystal was positioned at ϕ=0°
and ω=-28° and a set of 600 frames were collected. A series of 435 frames was
collected in the second shell with a starting position of ϕ=90° and ω=-28.0°. The crystal
was then moved to a position of ϕ=180° and ω=-28.0° to measure 230 frames required
for the third shell. The initial 50 frames of the first data shell were recollected at the end
of data collection to correct for any crystal decay, but none was observed.
Immediately after collection, the raw data frames were transferred to a second PC computer for integration by the SAINT program package (SAINT-NT V5.0, BRUKER AXS GMBH, D-76181 Karlsruhe, Germany, 2002). The background frame information was updated according to the equation $B' = (7B + C)/8$, where $B'$ is the update pixel value, $B$ is the background pixel value before updating, and $C$ is the pixel value in the current frame. The integration was also corrected for spatial distortion induced by the detector. In addition, pixels that reside outside the detector active area or behind the beam stop were masked during frame integration. The integrated intensities for the four shells of data were merged to one reflection file. The data file was filtered to reject outlier reflections. The rejection of a reflection was based on the disagreement between the intensity of the reflection and the average intensity of the symmetry equivalents to which the reflection belongs. In the case of strong reflections ($I > 99\sigma(I)$), which contains only two equivalents, the larger of the two equivalents was retains.

The structure was solved and refined by the SHELXTL software package (SHELXTL-NT V6.1, BRUKER AXS GMBH, D-76181 Karlsruhe, Germany, 2002).

**GC/MS Analysis.** GC/MS analysis was performed on a Hewlett-Packard (HP) 5890 Series II GC equipped with a HP5989A single quadruple mass spectrometer operating in EI mode, HP7673 autosampler, HP MS-DOS Chemstation, and HP-5MS capillary column (0.25 µm × 15 m × 0.25 mm). The oven temperature program was set as follows: initial temperature, 60°C for 3 min; gradient of 20°C/min until 140°C; gradient of 10°C/min until 200°C; hold time, 7 min; gradient of 20°C/min until 300°C; hold time, 10 min. The injection port was at 180°C; the source temperature was at 180°C; the temperature of the interface was at 280°C. The pressure of the carrier gas, helium, was set at 5 psi. For EI analysis, the ionization energy was 70 eV with a source pressure of $10^{-6}$ torr. For identification of permethylated glucuronide...
conjugate the injector temperature was set on 250°C. For GC/MS analysis 50 µl aliquot of the methanol fraction eluted from C-18 SPE cartridges was evaporated and reconstituted with 50 µl CHCl₃, of which 1 µl was injected into GC/MS.

**Derivatization Reactions.**

**Permethylation.** To the 50 µl aliquot of the methanol fraction eluted from C-18 SPE cartridges, evaporated to dryness under reduced pressure and redissolved in 50 µl of DMSO, 50 µl of CH₃I and 8-10 mg of powdered NaOH were added, and the reaction mixture stirred at room temperature for 3 min. One ml of H₂O was added and the products isolated by extraction with 2 ml of chloroform. The organic phase was subsequently washed with 3 ml of H₂O, dried over MgSO₄ and evaporated under reduced pressure. The dry residue redissolved in 50 µl of chloroform, of which 1 µl was injected to GC/MS.

**Silylation.** 50 µl aliquot of the methanol fraction eluted from C-18 SPE cartridges was evaporated to dryness under reduced pressure and the residue redissolved in 50 µl of anhydrous dichloromethane. The derivatization was performed at 60 °C by adding 50 µl of pyridine and 50 µl of trimethylchlorosilane and stirring for 30 min. The reaction was stopped by adding of 1 ml of H₂O and the products were extracted with 2 ml of chloroform. The organic phase was subsequently washed with 2 ml of H₂O, dried over MgSO₄ and evaporated under reduced pressure. The dry residues were redissolved in 50 µl of chloroform, of which 1 µl was injected into GC/MS.

**LC/MS Analysis.** LC/MS analysis of metabolites was carried out on HP1100 (Agilent Technologies, Walbron, Germany) HPLC system equipped with binary high-pressure mixing gradient pump, autosampler and column oven attached by electrospray interphase to a mass selective ion-trap detector (Esquire-LC, Bruker Daltonics, Bremen, Germany). The mass analyzer was interfaced with the LC using electrospray.
A positive ion MS mode was used with the following instrument parameters: nebulizer pressure, 40 psi; capillary voltage, 4500V; drying gas temperature, 350°C and drying gas flow, 5 L/min. Separation was performed on a reversed-phase column (Symmetry C18, 3.5 µm×2.1mm×100 mm, Waters Corp., MA). The initial mobile phase consisted of 0.01 mol/L ammonium formate and methanol. A linear gradient between 2 and 6 minutes increased the methanol concentration from 10% to 70%. The flow rate was 0.4 ml/min; the injection volume was 10 µl; and the column was maintained at ambient temperature. For LC/MS analysis, 50 µl of 0.01 M ammonium formate was added to 50 µl of the methanol fraction eluted from the C-18 SPE cartridge and 10 µl was injected into LC-MS.

PK Studies.

Analysis of TMCU in Plasma and Urine. Plasma and urine levels of TMCU were analyzed by GC/MS. The internal standard (valproyl glycinamide; 50 µl of 25 µg/ml solution in methanol) was added to the dry tubes and the organic solvent was evaporated under reduced pressure. 500 µl of plasma (or urine) and 100 µl of 1N NaOH solution were subsequently added to the tubes and mixed thoroughly. The extraction was performed with 2 ml of chloroform, which was subsequently evaporated under reduced pressure. The dry residues were reconstituted with 50 µl chloroform, of which 1 µl was injected into the GC/MS apparatus described above. The oven temperature program was set as follows: initial temperature, 60°C for 3 min; gradient of 20°C/min until 140°C; gradient of 10°C until 190°C; hold time, 3min. The injection port was at 180°C; the source temperature was at 180°C; the temperature of the interface was at 280°C. The pressure of the carrier gas, helium, was set at 5 psi. For EI analysis, the ionization energy was 70 eV with a source pressure of 10⁻⁶ torr. TMCU and the internal standard were monitored for selected
ions at m/z: 75, 97, 126, 129, 158, 169. Retention times of TMCU and internal standard were 9.2 min and 10.7 min, respectively. Calibration curves were constructed for each analytical run and were linear on the concentration range of 0.2-20 µg/ml. The developed method was validated according to the published guidelines (Shah et al., 2000). The quality control (QC) concentrations used to assess intra-day and inter-day precision and accuracy were within ±15% of the expected theoretical values.

Analysis of OH-TMCU in Plasma and Urine. Plasma and urine levels of OH-TMCU were analyzed by HPLC/UV system. The internal standard (levetiracetam; 100 µl of 1 mg/ml solution in methanol) was added to the dry tubes and the organic solvent was evaporated under reduced pressure. 500 µl of plasma (or 100 µl of urine) and 100 µl of 1N NaOH solution were added to the tubes and mixed thoroughly. The extraction was performed with 2 ml of tert-butyl methyl ether, which was subsequently evaporated under reduced pressure. The dry residues were reconstituted with 50 µl of mobile phase, of which 25 µl was injected into the HPLC/UV apparatus. Shimadzu HPLC system (model 10A) was equipped with C-18 column (LiChrCART, LiChrospher, 4 * 250 mm * 5 µm) and Shimadzu model SPD 10A UV detector set on 220 nm. Mobile phase consisted of 30% methanol in H2O and its flow was 0.7 ml/min. Retention times of internal standard and OH-TMCU were 6.2 min and 15.1 min, respectively. Calibration curves were constructed (using OH-TMCU standard methanol solution of 1 mg/ml) for each analytical run and were linear on the concentration range of 0.2-20 µg/ml in plasma and 5-500 µg/ml in urine. The developed method was validated according to the published guidelines (Shah et al., 2000). The quality control (QC) concentrations used to assess intra-day and inter-day precision and accuracy were within ±15% of the expected theoretical values.
Calculation of PK Parameters. The PK parameters were calculated by noncompartmental analysis based on statistical moment theory using PK software WinNonlin version 4.1 (Pharsight Co., Mountain View, CA).

Plasma Protein Binding. The plasma protein binding of TMCU was assessed at a concentration range of 5-150 mg/L. 2.5, 5, 25, 50 and 75 µg of TMCU were spiked with 0.5 ml of plasma. The test tubes vortexed vigorously for 1.5 min at 37°C and 50 µl aliquot was taken for evaluation of TMCU total plasma concentration by GC/MS method described above. The rest of the spiked plasma was transferred to the ultrafiltration tubes (Millipore, Bedford, MA) with 10 kDa cut-off. The tubes centrifuged at 4000g for 1.5 h at 37°C. TMCU unbound concentration in ultrafiltrate was assessed by GC/MS. TMCU fraction unbound in plasma (f_u) was calculated by dividing the unbound concentration in ultrafiltrate by the total plasma concentration.

Blood-to-Plasma Concentration Ratio. Blood-plasma partitioning of TMCU was determined by spiking 20, 40, 100, 200, 300, 400 and 800 µg of TMCU with 4 ml of fresh rat blood. The tubes vortexed vigorously for 1 min at 37°C, centrifuged at 3000g for 10 min, plasma was separated and analyzed for TMCU concentration by GC/MS. The blood-to-plasma concentration ratio (C_b/C_p) was calculated by dividing TMCU spiked concentration in blood by its analyzed concentration in plasma.

Chemical Stability in the Blood. Five test tubes containing 100 µg of TMCU were spiked with 3 ml of fresh rat blood. Following 1 min of vigorous vortex the tubes were transferred to the shaked bath at 37°C. After 2, 4, 6, 8 and 12 h the test tubes were withdrawn from the bath, centrifuged at 3000g for 10 min and evaluated for TMCU plasma concentration by GC/MS.

Water Solubility and LogP. Water solubility was assessed by stirring 125 mg of TMCU in 25 ml of H_2O at room temperature for 2 h. After stirring, the solution was
centrifuged at 3000g for 10 min, and 50 µl aliquot from supernatant was taken for evaluation of TMCU concentration by GC/MS.

For evaluation of logP (octanol-water partition coefficient) value of TMCU 1 ml, of aqueous potassium buffer at pH 7.4 and 1 ml of octanol were added to the test tubes containing 200, 300 and 400 µg of TMCU and mixed vigorously for 1 min. Mixing was continued at 37°C for 1 h, at which point 50 µl aliquot from the aqueous phase was taken for GC/MS analysis. Partitioning coefficient was calculated by dividing TMCU concentration in octanol by its concentration in aqueous buffer.
Results

Pharmacokinetics (PK). TMCU PK was studied following intravenous (5 mg/kg), oral (20 mg/kg) and intraperitoneal (20 mg/kg) administration to rats. TMCU fraction unbound (fu) was 52.8 ± 3.1% and was constant at a concentration range of 5-150 mg/L. TMCU was stable in whole blood over 12 h period and its blood-to-plasma concentration ratio was about 1 at a concentration range of 5-75 mg/L. TMCU had relatively low water solubility of 174 mg/L and its log P value was 2.24±0.08. The low water solubility of TMCU did not allow its i.v. administration at doses > 5 mg/kg.

TMCU concentration-time plots, as obtained following i.v., oral and i.p. dosing are presented in Fig. 2. TMCU concentration-time plot obtained following i.v. administration indicates that TMCU PK can be adequately described by a one-compartment open body model. TMCU PK parameters are presented in Table 1. Metabolic clearance (CL\textsubscript{m}) was a major component of TMCU total CL. Only a small fraction of 6.3 ± 0.8% was excreted as an unchanged drug in the urine. Due to relatively low liver extraction ratio (E = 10%), the value of intrinsic clearance was close to the value of unbound metabolic clearance. The \textit{MRT\textsubscript{iv}} of TMCU was 2.5 h, which is close to the expected value of a 1.44\textit{t}_{1/2} in the one-compartment open body model (\textit{MRT\textsubscript{iv}} = 1.44\textit{t}_{1/2}). TMCU concentration-time plots obtained following oral and i.p. dosing were characterized by multiple peak curves. It should be emphasized that oral administration of TMCU produced much more pronounced fluctuations in the C vs. t profile than the i.p. route. These fluctuations had an impact on the apparent terminal half-life of TMCU, which was elevated after oral dosing. Following i.p. dosing \textit{C\textsubscript{max}} was 15.4 mg/L and was reached 45 min after dosing. Following oral dosing \textit{C\textsubscript{max}} was only 6.5 mg/L and was reached after 4 h. In order to evaluate TMCU
absorption kinetics following these two extravascular modes of administration, Wagner-Nelson plots were constructed (Fig. 3) (Wagner and Nelson, 1964). Following i.p. dosing TMCU was absorbed very rapidly with 80% of the bioavailable drug being absorbed within 45 min. However, following oral dosing, 80% of the bioavailable drug was absorbed only after 6 h. In addition, the mean absorption time (MAT) values following oral and i.p. dosing were 3.1 h and 1.3 h, respectively. Despite a significant difference in the rate of absorption, the absolute bioavailability in both modes of administration was approximately the same: 83% and 90% following oral and i.p. dosing, respectively.

**Identification of Metabolites.**

Three metabolites of TMCU were identified in the rat urine, of which trans-2-hydroxymethyl-2,3,3-trimethylcyclopropanecarbonylurea (OH-TMCU) was a primary metabolite, and its sulfate and glucuronide conjugates were the two secondary metabolites (Fig. 4). OH-TMCU, the dominant metabolite of TMCU excreted in milligram quantities in the urine was isolated, crystallized and identified by NMR and X-ray crystallography (Table 2, Figs. 5,6). Sulfate and glucuronide of TMCU were excreted in the urine only in small quantities and it was difficult to isolate sufficient amounts of pure metabolites suitable for NMR analysis. These metabolites were identified by GC/MS and LC/MS (Tables 3&4, Figs. 7,8).

**NMR analysis of OH-TMCU.** Table 2 presents the complete assignment of the proton and carbon chemical shifts of the NMR spectra of OH-TMCU. The chemical shifts were assigned on the basis of the 2D spectra analysis (COSY, NOESY, HSQC and HMBC). The $^{13}$C NMR spectrum of OH-TMCU indicates that there are nine carbon atoms in the molecule (Fig. 5A). The two resonances at ~154 and ~173 ppm were assigned to carbonyl carbons (on the basis of their chemical shifts and intensities) and
the two weak resonances at ~36 and ~30 ppm belong to quaternary carbons that do not have any protons directly attached to them. The peak at ~65 ppm was assigned to a carbon atom bound to oxygen.

The proton NMR of OH-TMCU showed ten peaks (Fig. 5C and 5E). The three downfield peaks all have the same integration value, which was assumed to belong to a single proton. On the basis of this assumption, 16 protons could be counted in the NMR spectrum. The three singlet downfield resonances, in the 7-10 ppm range (Fig. 5C), were assigned to amide protons, on the basis of their chemical shifts, a temperature dependence study (not shown) and the lack of aromatic carbons in the $^{13}$C spectrum. The three most upfield resonances (3 protons each) clearly belong to three uncoupled methyl groups and the singlet at 1.43 ppm belongs to an uncoupled proton (Fig. 5E). The remaining three peaks at 3.28, 3.45 and 4.53 ppm belong to the same spin system (COSY – not shown) and their chemical shifts indicate their proximity to an oxygen atom.

Comparing the proton NMR spectrum of TMCU to that of OH-TMCU we see that the three most downfield peaks are identical in both compounds and can be assigned to the three amide protons (Fig. 5B and 5C). The upfield region of the TMCU (Fig. 5D) has only three peaks (in addition to the DMSO and the residual H$_2$O) which can be assigned as follows: the singlet at 1.32 ppm belongs to the methine and each of the two singlets at 1.15 and 1.10 ppm represents two magnetically equivalent methyl groups. The equivalence stems from the symmetry of the TMCU. In the upfield spectrum of OH-TMCU (Fig. 5E) there are three methyl peaks at 1.20, 1.18 and 1.16 ppm. This indicates that one of the methyl groups was modified and that now the molecule is no longer symmetric. The singlet at 1.43 ppm suggests that the methine is still intact. There are three more peaks, at 4.53 ppm (triplet, $\nu$=5.4 Hz), 3.45 ppm
(double doublet, \( j=11.15 \) and \( 5.4 \) Hz) and 3.28 ppm (double doublet, \( j=11.15 \) and \( 5.4 \) Hz). The chemical shifts and coupling constants suggest that the CH\(_3\) group has been oxidized to a CH\(_2\)OH group.

**X-ray crystallography of OH-TMCU.** X-ray crystallography of OH-TMCU single crystals revealed their exact spatial conformation (Fig. 6). Hydroxylation of trans-methyl group of TMCU results in formation of two chiral centers in the molecule with absolute (S) configuration at C1 and (R) configuration at C2.

**Identification of TMCU metabolites by GC/MS and LC/MS.** GC/MS retention time of OH-TMCU was 12.4 min and its electron ionization-mass spectrum (EI-MS) showed dominant fragment ions at m/z 55, 81, 97, 109 and 126, which were also the characteristic ones for the EI-MS of the parent compound (Fig. 7A, Table 3). Cleavage of C-C bond next to hydroxyl group yields m/z peak of 169 (Fig. 7A). LC/MS retention time of OH-TMCU was 9.2 min and its electrospray ionization (ESI) mass spectrum showed a pronounced ion peaks at m/z 201 and 223, which were [MH\(^+\)] and [M+Na\(^+\)] ions of OH-TMCU, respectively (Fig. 8, Table 4). In attempt to convert OH-TMCU to more volatile compound and simultaneously to improve the chances for surviving of the molecular ion, silylation and permethylation derivatizations were performed. EI of silylated OH-TMCU produced [M\(^+\)–CH\(_3\)] fragment ion at m/z 257 and EI of permethylated OH-TMCU produced a molecular ion [M\(^+\)] at m/z 256 (Table 3). GC/MS retention times of silylated and permethylated OH-TMCU were 11.6 min and 10.4 min, respectively.

TMCU sulfate was identified by GC/MS and LC/MS with retention times of 14.5 min and 8.7 min, respectively (Figs. 7B, 8, Tables 3&4). EI of sulfate conjugate yields the molecular ion at m/z 280 with characteristic for TMCU fragmentation (Fig. 7B). ESI produced m/z peak of 281, which was [MH\(^+\)] ion of sulfate conjugate (Table 4). EI-
MS of silylated sulfate conjugate of TMCU yielded a molecular ion at m/z 352 with GC/MS retention time of 15.4 min (Table 3).

Identification of underivatized TMCU glucuronide by GC/MS was relatively burdensome. LC/MS identification of the glucuronide resulted in small peak at retention time of 8.8 min, whose ESI yielded [MH⁺] ion at m/z 377 (Fig. 8, Table 4). Identification of permethylated glucuronide metabolites by GC/MS is common. Permethylated glucuronic acid moiety yields characteristic fragment ions at m/z 75, 101, 116, 141, 169, 201 and 232 (Thompson and Gerber, 1976; Lynn et al., 1978; Lynn et al., 1979). At the above-described conditions permethylated TMCU glucuronide was identified by GC/MS with retention time of 25 min. Its EI mass spectra showed characteristic above-mentioned fragments of permethylated glucuronic acid and (M+−CH₃) fragment ion at m/z 460 (Fig. 7C, Table 3).

**OH-TMCU kinetics following administration of the parent drug.** Following i.p. administration of 20 mg/kg TMCU, OH-TMCU was a major urinary metabolite. Pure OH-TMCU isolated from urine in crystalline form was utilized for preparation of standard solution serving for its quantitative evaluation in PK studies. Table 5 presents the PK parameters of OH-TMCU as obtained following i.v., oral and i.p. administration of TMCU. The t₁/₂ of OH-TMCU (3.6 h) was significantly longer than that of TMCU and it increased following extravascular dosing. Following i.v. administration of TMCU the MRT of OH-TMCU was 5.5 h and was increased after extravascular dosing. Analogously to the parent compound, C_max of OH-TMCU following i.p. dosing was higher than following oral dosing and was reached at the shorter time.

**Metabolic profile.** Following i.p. administration of 5 mg/kg of TMCU, 28.3 ± 2.6% of the dose was excreted as OH-TMCU in the urine. The Aₘ/FD (Aₘ⁻ cumulative
amount of OH-TMCU excreted in the urine) ratio increased to 45.6 ± 2.2% and 42.1 ± 3.8% following oral and i.p. administration of 20 mg/kg TMCU, respectively.
Discussion

The present study investigated TMCU pharmacokinetics and metabolic profile following its i.v., oral and i.p. administration to rats.

While TMCU's oral bioavailability was almost complete, it showed relatively slow and erratic absorption from the gastrointestinal (GI) tract probably due to its low water solubility. This resulted in a multiple peak pattern of the concentration-time plot (Fig. 2). TMCU was rapidly absorbed following i.p. administration, indicating that its relatively low water solubility may be compensated by high blood perfusion. Absorption of water insoluble drugs is dependent to a large extent on their migration from the stomach to the intestine. Precipitation of TMCU in the stomach after oral administration delays its movement toward the intestine where the total absorptive area and the blood perfusion are many times greater than in the stomach and where the membrane is much more permeable (Rowland and Tozer, 1995). Multiple peak pattern of TMCU concentration-time plot (Fig. 2) is most likely explained by the erratic migration of the precipitated drug from the stomach to the intestine where it is primarily absorbed. If the same phenomenon is observed in humans, the absorption kinetics of TMCU might be improved by enteric-coated formulation. The experiment performed with another water insoluble compound, phenylacetylurea, in which the small intestine of mice was tied up just below the stomach, showed that phenylacetylurea dose, which previously exerted anticonvulsant activity, was absolutely inactive at these experimental conditions (Everett and Richards, 1952).

The disposition kinetics of TMCU in rats can be adequately described by a one-compartment open body model, due to rapid equilibration of the drug between blood and tissues. The biopharmaceutical characteristics of TMCU prevented us from evaluating its PK linearity, since the maximal possible i.v. dose not precipitating in
the rats' vein was 5 mg/kg and extravascular administration of high doses was associated with incomplete and inconsistent absorption. The fu of TMCU was 52.8 ± 3.1% and was constant at a concentration range of 5-150 mg/L. TMCU volume of distribution (V ss = 0.87 L/kg) slightly exceeded the total body water of a rat, indicating little tissue sequestering.

TMCU undergoes extensive metabolism with only a small fraction excreted in the urine as an unmetabolized drug (6.3 ± 0.8%). Hydroxylation of the methyl group of 2,2,3,3-tetramethylcyclopropane moiety, to form OH-TMCU, was a major metabolic pathway of TMCU biotransformation in rats. Subsequently, OH-TMCU underwent sulfation and glucuronidation (Fig. 4). No TMCU was biotransformed to its corresponding acid, TMCA. Similar hydroxylation in rats of the methyl group of 2,2,3,3-tetramethylcyclopropane moiety in pyrethroid ester insecticides, fenpropathrin and terallethrin, had been previously reported (Mihara et al., 1981; Kaneko et al., 1987). Following i.p. administration of 5 mg/kg TMCU to rats, 28.3 ± 2.6% of the drug was excreted in the urine as OH-TMCU, while following i.p. administration of 20 mg/kg dose, 42.1 ± 3.8% was excreted in the urine as OH-TMCU. This phenomenon could be explained by a possible saturation of the sulfate conjugation pathway at higher doses. A similar observation was reported with paracetamol in humans (Clements et al., 1984). Sulfate conjugate of paracetamol was excreted in the urine at the extent of 38% and 33% following oral administration of 5 and 20 mg/kg doses, respectively. For a defined dose, the A m/D ratio was approximately the same after different routes of administration, indicating that the liver is probably the primary metabolic site for the biotransformation of TMCU to OH-TMCU. OH-TMCU was eliminated from the body slower (t 1/2=3.6 h) than the parent compound (t 1/2=1.6 h) (Tables 1 & 5), indicating that its elimination is not formation-rate limited.
As previously reported by our group (Sobol et al., 2004), oral administration of 30 mg/kg TMCU to rats resulted in relatively prolonged anticonvulsant protection, lasting up to 4 hours after dosing. Slow oral absorption of TMCU with a t_{\text{max}} of 4 hours may be a possible reason for such a long-lasting anticonvulsant activity.

In conclusion, TMCU undergoes rapid and almost complete absorption following i.p. administration to rats. Following oral administration the absorption of the drug is slow and erratic. TMCU disposition is adequately described by a one-compartment open body model. Oxidative metabolism is a major route of TMCU biotransformation. Only a small fraction of the administered dose is excreted in the urine as an unmetabolized drug. Hydroxylation of a methyl group of 2,2,3,3-tetramethylcyclopropane moiety is a major metabolic pathway of TMCU in rats. A portion of the primary metabolite, OH-TMCU, undergoes sulfation and glucuronidation.

The satisfactory PK profile, reported in the present study, along with broad and potent anticonvulsant activity of TMCU and positive feedbacks from the currently ongoing toxicological studies provide TMCU with a high potential for development as a new, potent and safe antiepileptic drug.

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References


Nau H (1986) Valproic acid teratogenicity in mice after various administration and phenobarbital-pretreatment regimens: the parent drug and not one of the metabolites assayed is implicated as teratogen. *Fundam Appl Toxicol* 6: 662-668.


Footnotes

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b) Professor Meir Bialer
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Jerusalem 91120, Israel

c) This work is abstracted from the PhD thesis of Mr. Eyal Sobol in partial fulfillment of the PhD degree requirements for The Hebrew University of Jerusalem.
Legends of figures

FIG. 1. Chemical structures of VPA, 2-ene VPA, 2,4-diene VPA, TMCA and TMCU.

FIG. 2. Plasma concentration-time plots of TMCU and its hydroxy metabolite (OH-TMCU) as obtained following i.v. (5 mg/kg, A), oral (20 mg/kg, B) and i.p. (20 mg/kg, C) administration of TMCU to rats.

FIG. 3. Wagner-Nelson plots for fraction of bioavailable drug absorbed following oral (A) and i.p. (B) administrations of TMCU (20 mg/kg) to rats.

FIG. 4. The metabolic scheme of TMCU in rats.

FIG. 5. The $^{13}$C NMR spectrum of OH-TMCU (A) and $^1$H NMR spectra of TMCU (B&D) and OH-TMCU (C&E).

FIG. 6. Drawing of trans-2-hydroxymethyl-2,3,3-trimethylcyclopropanecarbonylurea (OH-TMCU) from X-ray results.

FIG. 7. EI mass spectra of OH-TMCU (A), TMCU sulfate (B) and permethylated TMCU glucuronide (C).

FIG. 8. LC/MS chromatogram presenting OH-TMCU, TMCU sulfate and TMCU glucuronide peaks.
TABLE 1. PK parameters of TMCU as obtained following its i.v. (5 mg/kg), oral (20 mg/kg) and i.p. (20 mg/kg) administrations to rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>I.V. bolus</th>
<th>Oral administration</th>
<th>I.P. administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL (L/h·kg)</td>
<td>0.36</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CL/F (L/h·kg)</td>
<td>–</td>
<td>0.43</td>
<td>0.40</td>
</tr>
<tr>
<td>CLₚ (mL/h)</td>
<td>5.1</td>
<td>1.3</td>
<td>2.2</td>
</tr>
<tr>
<td>CLₘ (L/h·kg)</td>
<td>0.34</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CLₘᵢ (L/h·kg)</td>
<td>0.71</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vᵢ (L/kg)</td>
<td>0.82</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vᵢ/F (L/kg)</td>
<td>–</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Vₛₛ (L/kg)</td>
<td>0.87</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>t₁/₂ (h)</td>
<td>1.6</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>AUC (mg/L·h)</td>
<td>14.1</td>
<td>46.6</td>
<td>51.0</td>
</tr>
<tr>
<td>AUMC (mg/L·h²)</td>
<td>35</td>
<td>263</td>
<td>192</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.5</td>
<td>5.6</td>
<td>3.8</td>
</tr>
<tr>
<td>MAT (h)</td>
<td>–</td>
<td>3.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Cₘₕ (mg/L)</td>
<td>–</td>
<td>6.5</td>
<td>15.4</td>
</tr>
<tr>
<td>tₘₕ (h)</td>
<td>–</td>
<td>4</td>
<td>0.75</td>
</tr>
<tr>
<td>F (%)</td>
<td>–</td>
<td>83</td>
<td>90</td>
</tr>
<tr>
<td>E (%)</td>
<td>–</td>
<td>10.3</td>
<td>–</td>
</tr>
<tr>
<td>fe (%)</td>
<td>6.3 ± 0.8²</td>
<td>1.2 ± 0.5</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>Aₘ/D (Aₘ/FD)</td>
<td>29.4 ± 3.2</td>
<td>45.6 ± 2.2</td>
<td>42.1 ± 3.8</td>
</tr>
</tbody>
</table>

* Mean ± SD; CL-total clearance, CL/F-oral clearance, CLₚ-renal clearance, CLₘᵢ - metabolic clearance, CLₘᵢᵢ - intrinsic clearance, Vᵢ-volume of distribution based on linear terminal slope, Vᵢ/F-Vᵢ calculated after extravascular dosing, Vₛₛ-volume of distribution at steady state, t₁/₂-half-life, AUC-area under C vs. t plot, AUMC-area under Cₚₜ vs. t plot, MRT-mean residence time, MAT-mean absorption time, Cₘₕ-peak plasma concentration, tₘₕ-time to reach Cₘₕ, F-absolute bioavailability, E-liver extraction ratio, fe-fraction excreted unchanged in the urine, Aₘ-cumulative amount of OH-TMCU excreted in the urine.
TABLE 2. The complete assignment of the proton and carbon chemical shifts of the NMR spectra of OH-TMCU.\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
<th>C8</th>
<th>C9</th>
<th>O3</th>
<th>N1</th>
<th>N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H</td>
<td>1.43</td>
<td>NA\textsuperscript{b}</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>3.45</td>
<td>3.28</td>
<td>1.20</td>
<td>1.16</td>
<td>1.18</td>
<td>4.53</td>
<td>9.98</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>33.94</td>
<td>35.56</td>
<td>29.28</td>
<td>172.97</td>
<td>153.73</td>
<td>65.54</td>
<td>11.77</td>
<td>22.68</td>
<td>16.73</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The atoms were labeled according to the X-ray crystal structure appearing in Fig. 6

\textsuperscript{b} NA – not applicable
TABLE 3. Retention times and electron ionization mass spectral data of TMCU and its metabolites as obtained using GC/MS

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention time</th>
<th>Highest m/z ion (% abundance)</th>
<th>Five most abundant m/z ions (% abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMCU</td>
<td>9.2 min</td>
<td>184(1.6), M⁺</td>
<td>169(45), 126(40), 97(57), 81(44), 55(100)</td>
</tr>
<tr>
<td>OH-TMCU</td>
<td>12.4 min</td>
<td>169(15), M⁺−CH₂OH</td>
<td>152(46), 126(43), 97(53), 81(100), 55(93)</td>
</tr>
<tr>
<td>Trimethylsilylated OH-TMCU</td>
<td>11.6 min</td>
<td>257(1), M⁺−CH₃</td>
<td>215(9), 169(18), 130(54), 115(62), 73(100)</td>
</tr>
<tr>
<td>Permethylated OH-TMCU</td>
<td>10.4 min</td>
<td>256(0.1), M⁺</td>
<td>225(16), 142(74), 95(31), 85(29), 72(100)</td>
</tr>
<tr>
<td>TMCU sulfate</td>
<td>14.5 min</td>
<td>280(5), M⁺</td>
<td>109(19), 95(46), 81(74), 67(100), 55(72)</td>
</tr>
<tr>
<td>Trimethylsilylated TMCU sulfate</td>
<td>15.4 min</td>
<td>352(1), M⁺</td>
<td>129(31), 81(48), 75(100), 67(63), 55(52)</td>
</tr>
<tr>
<td>Permethylated TMCU glucuronide</td>
<td>25.0 min</td>
<td>460(1), M⁺−CH₃</td>
<td>232(89), 201(100), 141(46), 116(51), 101(48)</td>
</tr>
</tbody>
</table>
TABLE 4. Retention times and electrospray ionization mass spectral data of OH-TMCU, TMCU sulfate and TMCU glucuronide as obtained using LC/MS

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention time</th>
<th>[M+H]$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH-TMCU</td>
<td>9.2 min</td>
<td>201</td>
</tr>
<tr>
<td>TMCU sulfate</td>
<td>8.7 min</td>
<td>281</td>
</tr>
<tr>
<td>TMCU glucuronide</td>
<td>8.8 min</td>
<td>377</td>
</tr>
</tbody>
</table>
TABLE 5. Pharmacokinetic parameters of OH-TMCU obtained following i.v. (5 mg/kg), oral (20 mg/kg) and i.p. (20 mg/kg) administration of TMCU to rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>I.V. bolus</th>
<th>Oral administration</th>
<th>I.P. administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>3.6</td>
<td>4.3</td>
<td>3.9</td>
</tr>
<tr>
<td>AUC$_m$ (mg/L/h)</td>
<td>4.3</td>
<td>18.2</td>
<td>31.4</td>
</tr>
<tr>
<td>AUMC (mg/L/h$^2$)</td>
<td>23.3</td>
<td>174.5</td>
<td>253</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>5.5</td>
<td>9.6</td>
<td>8.1</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (mg/L)</td>
<td>0.7</td>
<td>1.6</td>
<td>3.6</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>1.0</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>AUC$_m$/AUC</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

$t_{1/2}$-half-life, AUC$_m$-area under $C$ vs. $t$ plot, AUMC-area under $C\cdot t$ vs. $t$ plot, MRT-mean residence time, $C_{\text{max}}$-peak plasma concentration, $t_{\text{max}}$-time to reach $C_{\text{max}}$, AUC-area under $C$ vs. $t$ plot of TMCU.
Figure 1
Figure 2

[Graph showing plasma concentration (mg/L) over time (h) for TMCU and OH-TMCU]
Figure 2

[Graph showing plasma concentration (mg/L) over time (h) for TMCU and OH-TMCU]
Figure 2
Figure 3

A

Fraction absorbed

Time (h)

0 2 4 6 8 10 12 14
Figure 3
Figure 4

TMCU

OH-TMCU

TMCU glucuronide

TMCU sulfate
Figure 5
Figure 5
Figure 5
Figure 6
Figure 7

[Diagram with peaks labeled with mass values and a chemical structure diagram labeled with OH, CONHCONH₂, and [M⁺-CH₂OH].]
Figure 7
Figure 7
Figure 8