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Article

Metabolic study of trimetazidine using ultra-high performance liquid chromatography-tandem mass spectrometry

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In the present study, the application of ultra-high performance liquid chromatography-tandem mass spectrometry allowed us to study of known—as well as hitherto unknown— trimetazidine (TMZ) metabolites in human urine and to propose their renal excretion profiles. Urine samples from a healthy volunteer were analyzed at baseline and at 0–4 h, 4–8 h, 8–12 h, and 12–24 h after a single dose of TMZ. A dilute-and-shoot procedure was used as sample treatment before separation. Full-scan spectra of possible metabolites were acquired. Additionally, product ion scan spectra of precursor ions of interest were also acquired at two collision energies. Intact TMZ was a major excretion product, with a maximum concentration at 4-8 h after administration. Moreover, five minor metabolites were observed, namely trimetazidine-N-oxide (M1), N-formyl trimetazidine (M2), desmethyl-trimetazidine O-sulfate (M3), desmethyl-trimetazidine O-glucuronide (M4), and desmethyl-trimetazidine-N-oxide-O-glucuronide (M5). Metabolite M5 has not previously been reported. Excretion curves were constructed based on the chromatographic peak areas of specific mass transitions (precursor ion > product ion) related to each of the detected metabolites.

Keywords: Trimetazidine. Liquid chromatography. Mass spectrometry. Excretion profiles. Metabolites.

INTRODUCTION

Trimetazidine (TMZ) is a piperazine-derived anti-angina agent with multiple cardioprotective effects, which include increasing coronary and blood circulation, reducing vascular resistance, promoting cardiac metabolism, and restoring energy production (Tsioufis, Andrikopoulos, Manolis, 2015; Dézsi, 2016). The mechanism of action of TMZ is uncertain, but the drug may improve celular tolerance to ischemia by inhibiting mitochondrial enzyme 3-ketoacyl coenzyme A thiolase (3-KAT), thus reducing myocardial free fatty acid oxidation and increasing glucose oxidation. Stimulation of glucose oxidation requires less oxygen consumption, which may explain the cardioprotective effect of TMZ (Kantor *et al.,* 2000; Marzilli, 2003). The addition of TMZ to exercise training in patients with ischemic heart disease and left ventricular dysfunction could significantly improve functional capacity, left ventricular ejection fraction, and endothelium-dependent dilatation (Belardinelli *et al.,* 2008). In recent years, TMZ has emerged as a novel option for the treatment of heart failure in patients with an ischemic etiology, diabetes, or obesity (Winter *et al.,* 2014). Based on research results and a meta-analysis of controlled trials on the use of TMZ for the treatment of cardiomyopathy and heart failure, the European Society of Cardiology (Lopatin, 2016) included TMZ in their 2016 guidelines for the treatment of patients with angina and heart failure with reduced ejection fraction (Fragasso *et al.,* 2006; Huang, Dong, 2007; Gao *et al.,* 2011; Hu *et al.,* 2011; Zhang *et al.,* 2012; Fan, Niu, Ma, 2018). Due to its possible performance-enhancing effect, TMZ was also added to the

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World Anti-Doping Agency (WADA) Prohibited List on January 1, 2014 as a substance prohibited in competition (World Anti-Doping Agency, 2014). Originally, TMZ was included in group S 6.B (Specified stimulants), based on the similarity of its chemical structure to some of the listed stimulants. Nowadays, TMZ is listed in section 4.4 as a metabolic modulator, prohibited in- and out-of-competition (World Anti-Doping Agency, 2021).

Several analytical methods have been reported for monitoring urinary TMZ levels during treatment and for routine doping control analysis. For example, Barré *et al.* (2003) determined TMZ in urine by gas chromatography (GC) coupled with a nitrogen-phosphorus selective detector, while Fay *et al.* developed a quantitative method using GC/mass spectrometry (MS). The disadvantage of both methods is the time-consuming derivatization step necessary to ensure adequate volatility. To determine TMZ in pharmaceutical formulations and human urine, Ghoneim *et al.* proposed the use of a square wave adsorptive stripping voltammetry procedure at a glassy carbon electrode.

Other authors preferred liquid chromatography coupled with a photodiode array detector (El-Alfy *et al.,* 2019) or mainly with MS (Jackson *et al.,* 1996; Sigmund *et al.,* 2014), which is nowadays the method of choice for TMZ analysis. In most anti-doping methods, TMZ is monitored as the parent compound in multiresidue methods alongside other families of compounds such as stimulants, β2-agonists, or diuretics (Dong *et al.,* 2015). For sample treatment, solid phase extraction with C18 cartridges (Jackson *et al.,* 1996) or dilute-and-shoot methods (Sigmund *et al.,* 2014; Dong *et al.,* 2015) are preferred. To the best of our knowledge, only 2 studies have focused on the characterization of TMZ metabolites in human urine. Although TMZ is excreted in the urine mainly unchanged, as confirmed by both of the above-mentioned studies, differences were identified in the metabolites (Jackson *et al.,* 1996; Sigmund *et al.,* 2014).

Therefore, the aim of this work was to screen, identify and confirm reported and unreported urinary TMZ metabolites after administration of a single dose and to propose their renal excretion profiles. To this end, we used ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS), since it is a powerful technique with excellent sensitivity and selectivity and is able to identify non-target compounds by means of high-resolution and accurate mass measurements.

MATERIAL AND METHODS

Chemicals and reagents

This work used the following chemicals: TMZ reference standard (European Pharmacopoeia), diphenylamine, used as internal standard in positive ionization mode (Sigma-Aldrich, Saint-Louis, USA), piretanide impurity A, used as internal standard in negative ionization mode (European Pharmacopoeia), LC-MS-grade methanol (Fisher Scientific, Hampton USA), LC-MS-grade acetonitrile (Sigma-Aldrich, Saint-Louis, USA), ammonium formate (VWR Chemicals, Radnor, USA), and formic acid (Fluka, Buchs, Switzerland). Ultrapure water was prepared by a Milli-Q purification system (Millipore Ibérica, Madrid, Spain).

Excretion study samples

A healthy volunteer (male, 60 years old, 95 kg) was administered a single oral dose of 20 mg TMZ (IDAPTAN 20 mg, Lot N-011). Urine samples were collected at baseline and from 0–4 h, 4–8 h, 8–12 h, and 12–24 h after administration. The volunteer gave his informed consent for participation in the study according to the principles of the Declaration of Helsinki. The study was conducted in accordance with the Good Clinical Practice Guidelines of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use. Ethics approval was granted by the Ethics Committee of our Institute (Consorci Mar Parc de Salut de Barcelona, Spain; 2012/4981) and the Spanish Medicines Agency (EudraCT protocol number 2010-002288-80).

Preparation of standard solutions

A Stock standard solution of TMZ was prepared from TMZ standard powder in methanol at a concentration of 100 $\mu g.mL^{-1}$ and stored at −20°C. Working standard solutions were prepared from stock solution by appropriate dilution with methanol. Stock

solutions of 100 μg.mL^{-1} of the internal standards were prepared in the same way.

Sample preparation

Urine samples (one 0.5-mL aliquot) were centrifuged at 12,000 rpm for 5 minutes and then 300 μL of the supernatant were diluted 1:5 with a solvent composed of ultrapure water: acetonitrile (99:1) with 1% formic acid, containing the internal standards at concentrations of 100 ng.mL^{-1} (Piretanide Impurity A) and 20 ng.mL^{-1} (Diphenylamine). For quantitative estimation of TMZ in urinary excretion samples, 2 positive controls were prepared by spiking a blank urine at 20 ng.mL^{-1} and at 200 ng.mL^{-1}. These positive control samples, as well as negative control samples, were processed and analyzed together with the excretion study samples.

Method validation

Several method quality parameters were evaluated for method validation. The intraday precision of the method was determined by analyzing 6 blank urine spiked with TMZ at 10 ng. mL^{-1} (low concentration level) and 50 ng.mL^{-1} (high concentration level). Assay precision was expressed as relative standard deviation (%RSD). The limit of detection was extrapolated from the low concentration samples as the concentration that would provide a signal-to-noise ratio of 3.

Matrix effects, defined as direct or indirect alteration in response to the presence of interfering substances in the sample, were evaluated by comparing the analyte peak areas of 6 blank urine samples from different volunteers spiked with TMZ with the peak areas of ultrapure water spiked with TMZ at the same concentration level of 10 ng.mL^{-1}.

The selectivity of the method was investigated by evaluating chromatograms of the blank urine samples obtained from the six volunteers, to ensure they were free of interferences at the retention time of TMZ.

Instrumentation and analysis conditions

The UHPLC-HRMS analysis was performed by using a Q-Exactive (Thermo Scientific, Bremen, Germany) quadrupole-orbitrap mass spectrometer equipped with an electrospray (ESI) ionization source operated in positive and negative modes and coupled with a Vanquish Flex (Thermo Scientific, Bremen, Germany) binary UHPLC system. Chromatographic separation was achieved on column Acquity UPLC BEH C18 (2.1 x 100 mm, 1.7 μm particle size) (Waters, Milford, USA) using a mobile phase composed of 0.1% formic acid and 1.0 mM ammonium formate in methanol (solvent A) and 0.1% formic acid and 1.0 mM ammonium formate in water (solvent B). The gradient elution employed was as follows: 0-2.5 min, 100% B; 2.5-5.5 min, 97% B; 5.5-5.8 min, 10 % B; 5.8-8.1 min, 3 % B; 8.1-11.00 min, 100% B. The column temperature was maintained at 45°C during the chromatographic run, a flow rate of 0.3 mL/min was used, and the injection volume was 10 μL. The spray voltage of the ESI source in negative and positive ion mode was 3kV with capillary temperature 320°C.

Mass spectrometry data were acquired in full-scan mode throughout the *m/z* range 63-750 at a resolution of 35,000 full width half maximum (FWHM) at *m/z* 200. To acquire more information about fragmentation of molecular ions of TMZ and its metabolites, reinjection of the sample extracts in product ion scan (Parallel Reaction Monitoring, PRM) modes was performed also at a resolution of 35,000 FWHM and with an isolation width of 1.5 *m/z,* at normalized collision energies (NCE) of 15 and 45 eV. All the data recording and processing were acquired with Thermo Xcalibur 2.1 SP1 and TraceFinder 4.2 software.

RESULTS AND DISCUSSION

Method validation

The method was validated for TMZ in terms of selectivity, precision, limit of detection (LOD) and matrix effects. None of the blank urine samples tested showed interferences at the TMZ retention time. Intraday precision was 2.8% for the low concentration level and 17.7% for the high concentration level, which was acceptable for the concentration range tested. LOD was estimated at 1 ng.mL^{-1}, which is well below half of the minimum required performance level of 20 ng.mL^{-1}

established by WADA. Matrix effects were calculated as the signal response in the urine samples divided by the average signal response in the water samples, expressed as a percentage. Thus, values above 100% denote signal enhancement whereas values below 100% represent signal suppression. Generally, slight signal suppression was observed, with values ranging from 43.2% to 62.7%, with an average value of 50.8% (n=6). Variability of matrix effects, expressed as %RSD, was 15%, corresponding to low variability of matrix effects. Since the objective of this work was not to provide accurate quantitation but rather to estimate the excreted concentration, and the variation of matrix effects among the various matrices studied was low, it was assumed that acceptable estimates of the TMZ concentration could be performed without the need for correction with a deuterated internal standard. Overall, the evaluated quality parameters showed acceptable results and the method was considered valid for screening purposes.

Strategies for the detection of TMZ metabolites

After a single dose, TMZ is predominantly eliminated in the urine with an elimination half-life of 6.5 h, without a cumulative dose effect (Ciapponi, Pizarro, Harrison, 2017). Due to its physicochemical properties, TMZ and its metabolites can be retained and separated in a C18 stationary phase by reversed-phase chromatography (Zhou *et al.,* 2010). In this work, a UHPLC C18 column was combined with a mobile phase gradient starting at 100% aqueous conditions to ensure the retention of highly polar metabolites such as glucuronides. Moreover, the addition of a low concentration of ammonium formate buffer might help with the retention of sulfates by the formation of ion pairs.

A previously reported study of TMZ metabolism using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) applied multiple reaction monitoring (MRM) mode (Jackson *et al.,* 1996). The most complicated step in performing the MRM method is to carefuly study the fragmentation of the precursor ions (Picotti, Aebersold, 2012). Moreover, previous information on the metabolite and its fragmentation pathways is needed to adequately select the MRM transitions. Another study used product ion scan spectra acquired in HRMS to characterize the metabolites according to the literature (Sigmund *et al.,* 2014).

In this work, a database was created, assuming metabolic regularities of possible phase I and phase II transformations of TMZ, adding up to 60 possible metabolites. The accurate mass of possible protonated/deprotonated ions of TMZ metabolites from the database was calculated and the resulting table was used in a target processing method to identify possible candidates in the full-scan data of the post-administration samples that were not present in the basal samples using TraceFinder software. To provide additional information for the identification of the candidates compatible with possible TMZ metabolites, sample extracts were reinjected with application of PRM mode. Precursor ions were selected for fragmentation at low and high collision energies (15 eV, 45 eV) and their product ion scan spectra were compared with that of TMZ and with those of other metabolites reported in the literature. Collision induced dissociation (CID) spectra of intact TMZ were obtained as a major unchanged metabolite of the administered drug and 5 minor possible TMZ metabolites. TMZ and four possible candidates were detected in positive ionization mode, while only one additional candidate was detected in negative ionization mode. Accurate mass and the elemental composition of TMZ and detected metabolites, precursor ions and the most abundant product ions are listed in Table I.

TABLE I - Elemental compositions of ionized molecules of detected metabolites and resulting product ions using high-resolution/high accuracy MS^n

Compound	Molecular mass	Precursor ion (*m/z*)	Polarity	Molecular formula (exp.*)	Error (ppm)	Retention time (min.)	Collision energy (eV)	Product ions (*m/z*)	Molecular formula (exp.*)	Error (ppm)	Metabolic pathway
TMZ	266	267.17004	+	$C_{14}H_{23}O_3N_2$	-1.04	4.88	45	181.08565	$C_{10}H_{13}O_3$	-1.50	parent compound
								166.06234	$C_9H_{10}O_3$	-0.64	
								136.05184	$C_8H_8O_2$	-0.30	
M1	282	283.16544	+	$C_{14}H_{23}O_4N_2$	0.73	4.78	45	181.08572	$C_{10}H_{13}O_3$	-1.11	oxidation
								166.06244	$C_9H_{10}O_3$	-0.04	
								136.05189	$C_8H_8O_2$	0.07	
M2	294	295.16461	+	$C_{15}H_{23}O_4N_2$	-2.11	5.04	45	181.08579	$C_{10}H_{13}O_3$	-0.39	conjugation
								166.06235	$C_9H_{10}O_3$	-0.58	
								136.05185	$C_8H_8O_2$	-0.23	
M3	332	331.09692	-	$C_{13}H_{19}O_6N_2S$	-0.03	3.80	45	251.14000	$C_{13}H_{19}O_3N_2$	-0.46	conjugation
								236.11621	$C_{12}H_{16}O_3N_2$	-1.83	
								221.09278	$C_{11}H_{13}O_3N_2$	-1.75	
								79.95596	SO_3	-17.55	
M4	428	429.18710	+	$C_{19}H_{29}O_9N_2$	0.80	1.79	45	167.07038	$C_9H_{11}O_3$	0.65	conjugation
								139.07547	$C_8H_{11}O_2$	0.82	
								107.04963	C_7H_7O	4.57	
							15	87.09237	$C_4H_{11}N_2$	7.98	
M5	444	445.18108	+	$C_{19}H_{29}O_{10}N_2$	-1.33	4.26	45	167.07038	$C_9H_{11}O_3$	0.65	oxidation + conjugation
								139.07533	$C_8H_{11}O_2$	-0.19	
								107.04950	C_7H_7O	3.35	

Characterization of detected metabolites

No peaks were observed at the same retention time in pre-administration samples (Figure 1). Evaluation of the data obtained from the product ion mass spectra acquired in positive and/or negative ionization mode revealed the structures of previously reported TMZ metabolites detected, as well as a that of hitherto undetected and unreported metabolite (Figure 2, Figure 3).

For most of the detected metabolites in this work, chemical structures only are proposed. For the complete characterization of these metabolites, synthesis of the reference materials and subsequent characterization by nuclear magnetic resonance or crystallographic studies are required.

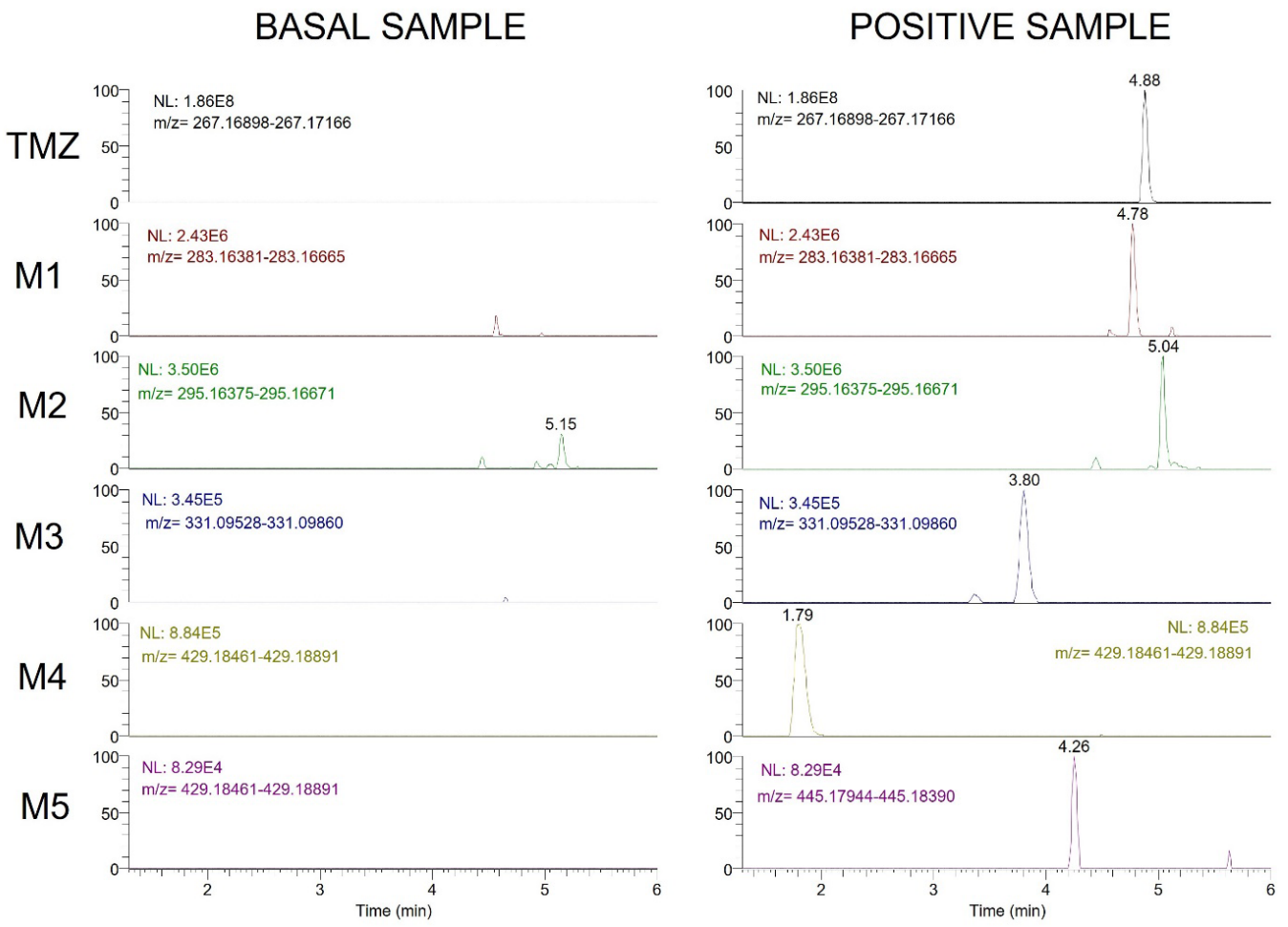

FIGURE 1 - Chromatograms of the basal urine sample and sample containing TMZ and its metabolites M1-M5.

TMZ = trimetazidine; M1 = trimetazidine-N-oxide; M2 = N-formyl trimetazidine; M3 = desmethyl-trimetazidine O-sulfate; M4 = desmethyl-trimetazidine O-glucuronide; M5 = desmethyl-trimetazidine-N-oxide-O-glucuronide.

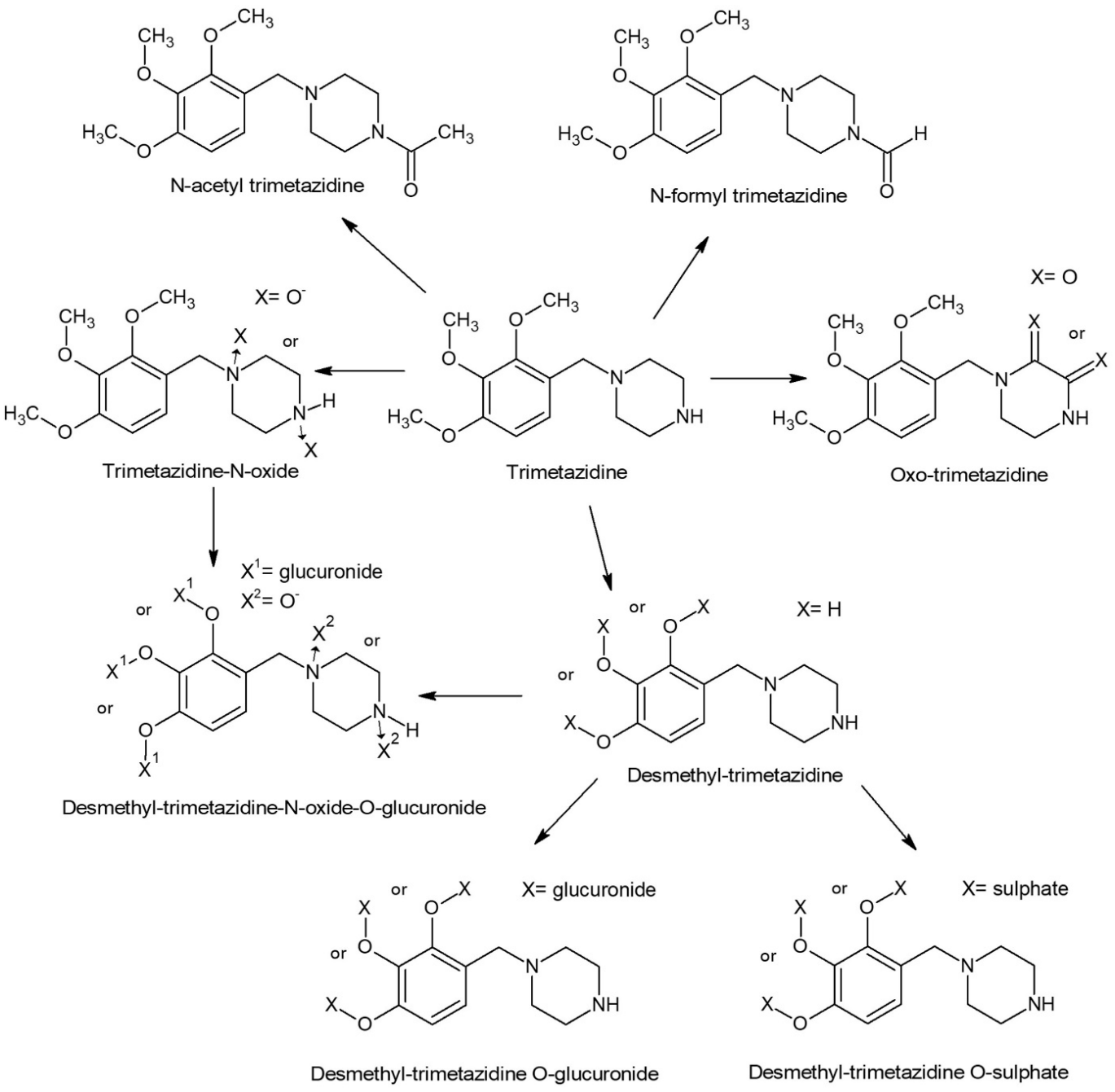

FIGURE 2 - Proposed metabolic pathways of trimetazidine.

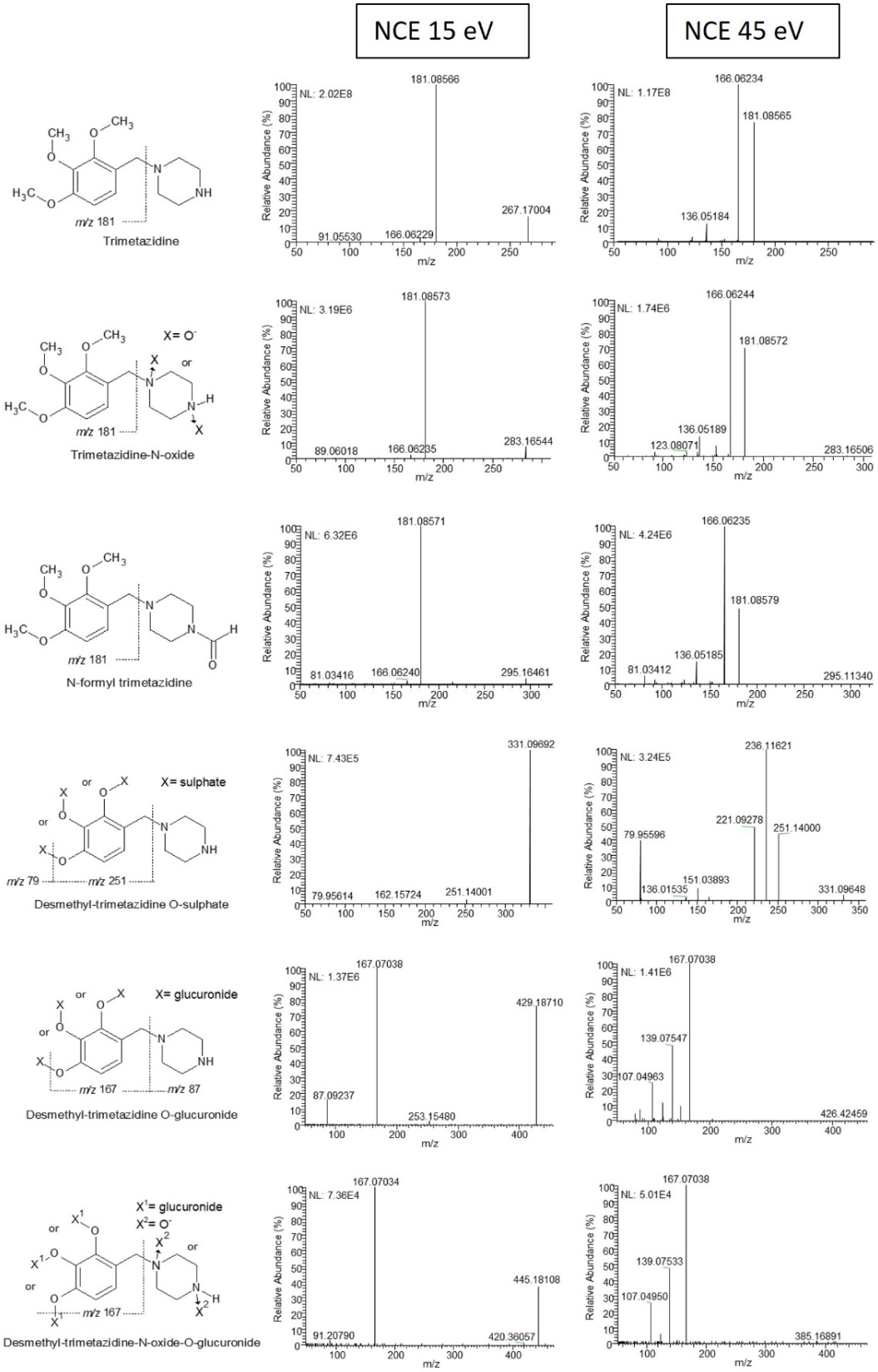

FIGURE 3 - Product ion mass spectra of ionized molecules of TMZ and its metabolites (trimetazidine-N-oxide, N-formyl trimetazidine, desmethyl-trimetazidine O-sulfate, desmethyl-trimetazidine O-glucuronide, desmethyl-trimetazidine-N-oxide-O-glucuronide).

Intact TMZ

Among all the detected substances, TMZ was the only standard available as a certified reference standard for its confirmation and quantification in urine samples. Intact TMZ was detected and confirmed by comparison with the product ion scan spectra in all the post-administration samples. TMZ was observed at very high abundances in comparison to the other observed metabolites (Figure 4). Although it can not be supposed that the response factor of TMZ and its metabolites is comparable, based on the signal observed, most of the drug was excreted in the urine unchanged, as already reported in the literature (Dézsi, 2016).

The fragmentation pattern of TMZ has been reported in the literature (Jackson *et al.,* 1996; Sigmund *et al.,* 2014). The experimental product ion scan of TMZ was compared with that of the literature to identify the product ions to be used as a reference for identification of other metabolites. The fragmentation of $[M+H]^+$ of TMZ at *m/z* 267 at low collision energies (NCE 15 eV) (Figure 3) predominantly showed the product ion at *m/z* 181, which Sigmund *et al.* (2014) suggested to result from the elimination of the piperazine ring (loss of 86 Da). Other detected product ions at high collision energies (NCE 45 eV) at *m/z* 166 and 136 were assigned to the elimination of a methyl radical (loss of 15 Da) and formaldehyde. The elemental composition of the product ions was confirmed by the determination of their accurate mass and was consistent with previously published data.

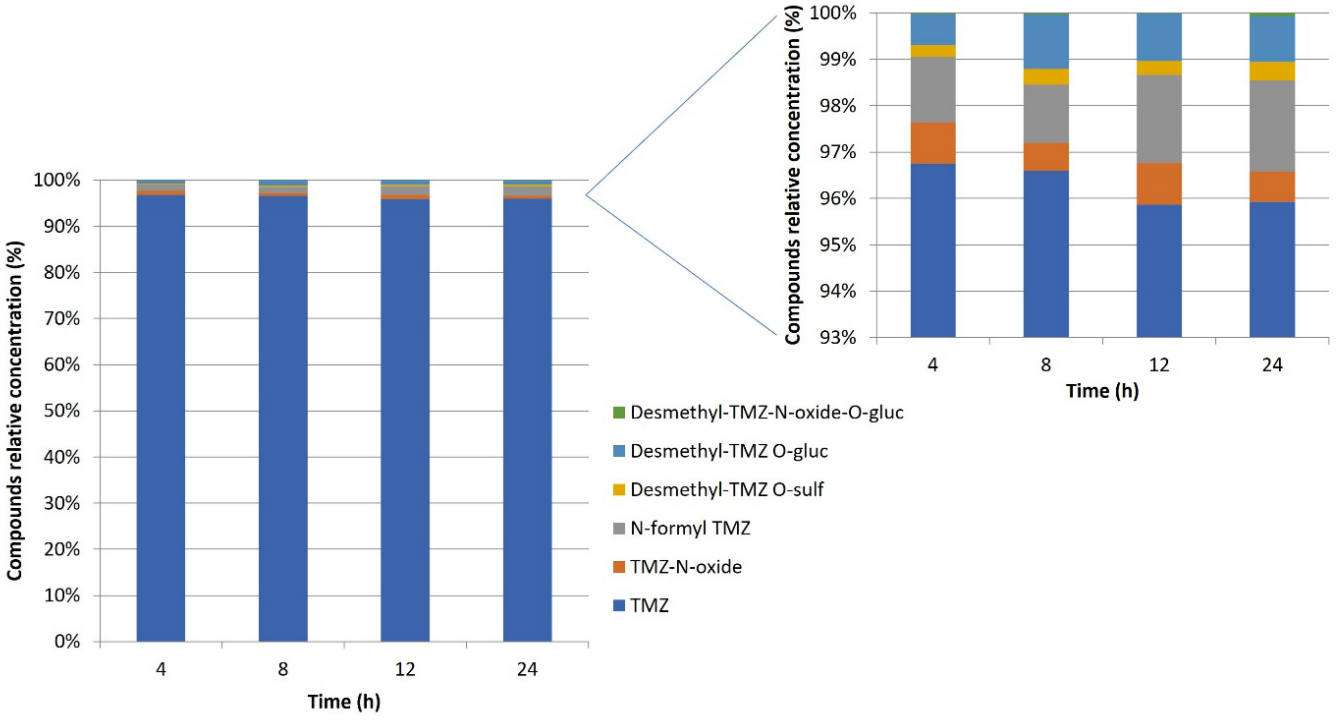

FIGURE 4 - Relative concentrations of TMZ and its metabolites in urine at 4 h, 8 h, 12h, and 24 h after a single dose of TMZ.

M1 and M2 metabolites

Both M1 and M2 metabolites were ionized and detected only in positive ionization mode at *m/z* 283 (M1) and 295 (M2). Moreover, they shared the same main product ions in the CID spectra (*m/z* 181, 166 and 136), which perfectly matched the data obtained for intact TMZ. The elemental composition and mass spectra of the detected metabolites M1 and M2 (Table I, Figure 3) suggested that oxidation or conjugation with formic

acid is located in the nitrogen atom at the piperazine ring, since the same product ions at *m/z* 181, 166 and 136 arise from the unmodified part of the structure. This interpretation, together with the exact mass, are in agreement with previously reported data (Jackson *et al.,* 1996; Sigmund *et al.,* 2014). Thus, the M1 metabolite was identified as a phase I metabolite TMZ-N-oxide formed by oxidation of TMZ and M2 as N-formyl TMZ formed by conjugation of TMZ with formic acid.

Recently, an impurity was reported in the trimetazidine dihydrochloride tablet formulation, identified as N-formyl trimet azidine, which corresponds to the structure of the M2 metabolite (Jefri *et al.,* 2018). The experiments confirmed the identity of the impurity and the possible mechanism of its formation, which involved reaction of the drug with residual formic acid present in the tablet binder used in the formula (Jefri *et al.,* 2018). Thus, there is a suspicion that the M2 metabolite detected at *m/z* 295 and tentatively identified as N-formyl trimetazidine was not a true product of the metabolic pathway of TMZ but was rather an impurity of the trimetazidine dihydrochloride tablet formulation excreted unchanged. To confirm this hypothesis, further studies are needed.

M3 and M4 metabolites

The molecular mass (MM) of M3 (332 Da) suggested desmethylation as phase I metabolism before conjugation with sulfate. The M3 metabolite was the only compound detected in negative ionization mode, which is also in agreement with it being a sulfate metabolite. The product ion scan mass spectrum yielded the most abundant ions at *m/z* 251, 236, 221 and 80 (Figure 3), which were assigned to the loss of SO_3 (*m/z* 251), followed by successive losses of radical methyl from the previous ion (*m/z* 236 and *m/z* 221) and the $[SO_3]^-$ ion (*m/z* 80), which is characteristic of sulfate metabolites. Of note, the very high error (Table I) observed for the assignment of the ion at *m/*z 80 was probably due to the calibration being made with a commercial mixture, which has a mass range beginning at *m/z* 265 (dodecylsulphate). However, the assignment could be done with sufficient certainty, based on the characteristic isotope cluster due to the sulfur atom and the lack of other possible combinations arising from the composition of the parent compound. An additional product ion at *m/z* 151 with very low relative intensity could be assigned to the loss of piperazine moiety from the ion at *m/z* 236. The assignment of the product ions observed in the product ion scan spectra was consistent with a structure similar to desmethyl-trimetazidine O-sulfate.

This metabolite has previously been reported only in positive ionization mode (as a $[M+H]^+$ ion at *m/z* 333), with product ions at *m/z* 253, 167 and 87 (Jackson *et al.,* 1996; Sigmund *et al.,* 2014). However, the product ion scan spectra are not directly comparable as they are obtained in different polarities and from different precursor ions. Nevertheless, product ion at *m/z* 251 in negative ionization mode could be equivalent to product ion at *m/z* 253 in positive ionization mode, both arising from elimination of sulfate moiety (*m/z* 80). Therefore, the identification of this metabolite as desmethyl-trimetazidine O-sulfate is consistent with the literature.

The MM of M4 was 428 Da, and was detected as the $[M+H]^+$ ion in positive ionization mode. Its product ion scan (Figure 3) showed the product ions at *m/z* 167, 139, 107 (NCE 45 eV) and an abundant product ion at *m/z* 87 (NCE 15 eV). The same product ions were found for desmethyl-trimetazidine collision induced dissociation spectra in previous works in the literature (Jackson *et al.,* 1996; Sigmund *et al.,* 2014). According to these data, M4 was tentatively identified as desmethyl-trimetazidine O-glucuronide, considered to be formed by TMZ demethylation as phase I metabolism followed by glucuronide conjugation. Thus, fragmentation of M4 may begin with the concurrent losses of piperazine (86 Da) and the glucuronide moiety, and then follow the fragmentation pathways of the unconjugated metabolite described in the literature.

It has been proposed that TMZ demethylation is a major metabolic reaction leading to 2-desmethyl- and 3/4-desmethyl-trimetazidine, with 2-desmethyl-trimetazidine being a minor metabolite (Jackson *et al.,* 1996). However, in our study, unconjugated demethylated TMZ metabolites were not detected in any of the samples, and we observed only these M3 and M4 conjugated metabolites of demethylated TMZ.

M5 metabolite

The MM of this metabolite was 444 Da, and was detected in positive ionization mode as a $[M+H]^+$ ion. According to its accurate mass, it differed in one atom of oxygen from M4. The dissociation behavior of M5 (Figure 3) yielded the same major product ions as M4 (*m/z* 167, 139, 107 at NCE 45 eV) except for the product ion at *m/z* 87, which was absent. This observation might indicate that the M5 metabolite resulted from N-oxidation or hydroxylation conducted in the piperazine moiety. The M5 metabolite could be formed by oxidation through the nitrogen atom or hydroxylation at the piperazine ring of desmethyl-trimetazidine (after desmethylation or at the same time) as phase I metabolism steps followed by conjugation with glucuronic acid. Since trimetazidine-N-oxide is a previously known TMZ metabolite (Sigmund *et al.,* 2014) and no hydroxylated metabolites have yet been described (Jackson *et al.,* 1996; Sigmund *et al.,* 2014), the most probable structure for the M5 metabolite based on its elemental composition and the information obtained from the product ion mass spectrum is desmethyl-trimetazidine-N-oxide-O-glucuronide, which is a hitherto unreported metabolite of TMZ. However, further experiments are needed to confirm this assignment.

Renal excretion study

The TMZ elimination experiments in a single healthy volunteer enabled us to follow the changes in urinary TMZ concentrations at four different time points after administration of a single dose. Although TMZ was the only compound that had a certified reference standard for its quantification, comparison of its peak area with areas of M1-M5 metabolites (Figure 4) allowed us to assume that TMZ is mainly excreted as an intact drug. TMZ concentrations were estimated using response factors from spiked urine samples and the concentration was corrected by specific gravity. Its elimination profile was constructed (Figure 5). As can be seen, TMZ seemed to follow a fast elimination profile, with a peak concentration at 8 h after administration. Unfortunately, the detection window for TMZ could not be estimated because only samples from the first 24 h were available. The maximum TMZ concentration (11.4 $\mu g.mL^{-1}$) in urine samples occurred 4-8 h after administration. This is in agreement with previously reported data (Jarek *et al.,* 2014).

Despite the impossibility of performing a quantitative analysis of TMZ metabolites (due to the lack of available analytical standards), the detector response in MS analysis can be considered to be proportional to the metabolite concentrations in the urine samples, since their structures are closely related. Thus, pharmacokinetic curves were constructed by comparing the chromatographic peak areas associated with each *m/z* value of the M1-M5 metabolites with their maximum peak area (Figure 6). As can be seen, almost all metabolites followed the same behavior as TMZ. M5 showed a slightly different profile but this could be attributed to higher variability in the signal due to the low response for this metabolite.

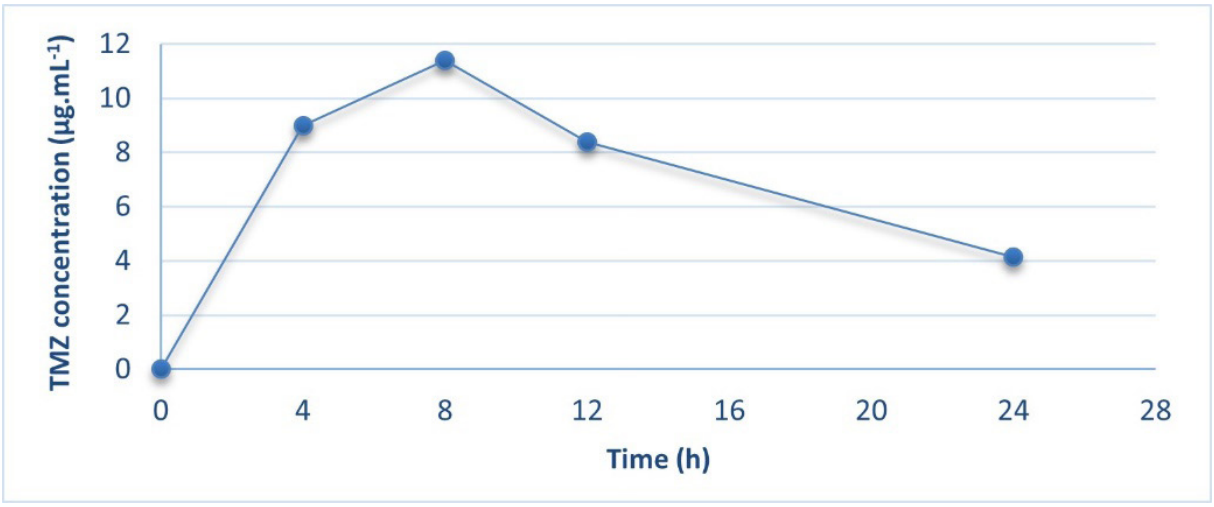

FIGURE 5 - TMZ Excretion profile.

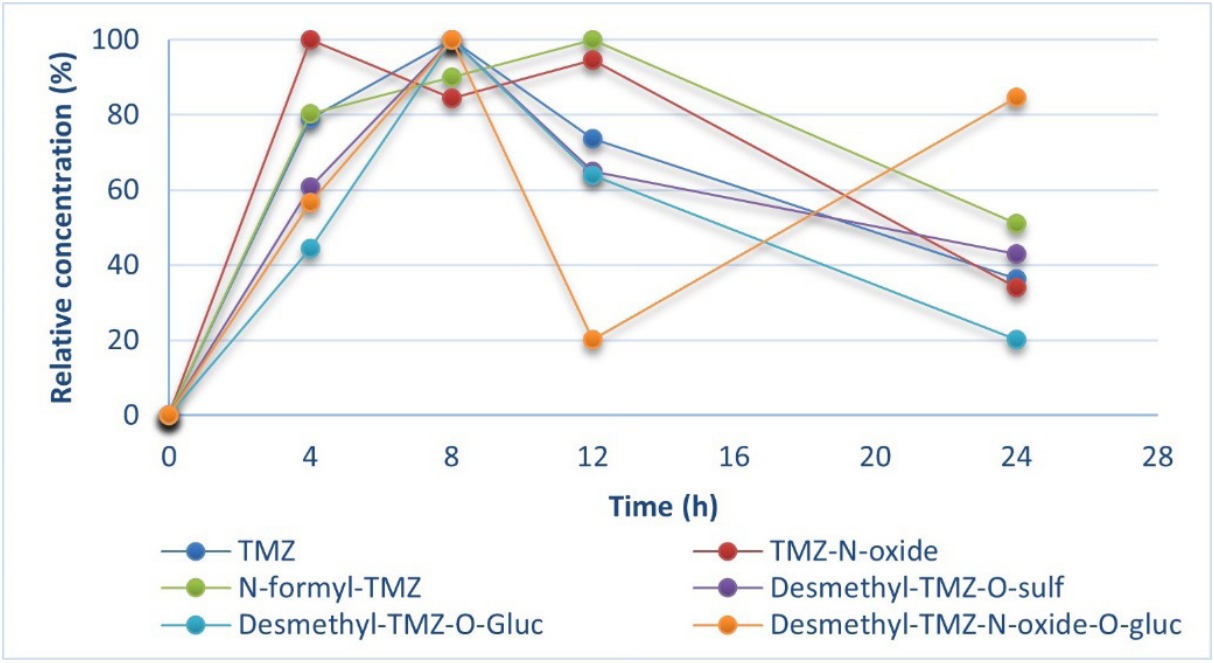

FIGURE 6 - Proposed excretion profile of TMZ and its metabolites.

CONCLUSIONS

In this study, TMZ and 5 metabolites were detected in human urine by LC-MS/MS analyses in positive and negative ionization mode. A combination of high selectivity in the quadrupole MS and high-resolution in the Orbitrap MS demonstrates that this is a highly effective method for metabolic study of TMZ in complex matrices. A new metabolite has been described. The method had good sensitivity and specificity and we observed no significant interferences or matrix effect caused by endogenous compounds. Moreover, this study discusses renal excretion of intact TMZ and proposes excretion profiles of minor metabolites that could be useful to study other drugs with a similar chemical structure (e.g. lomerizine).

In summary, the results of the present study demonstrate that following oral TMZ administration, most of the drug was excreted in the urine unchanged. Therefore, the method is suitable for TMZ determination, and can also

be used in doping control analysis or as a therapeutic drug monitoring method to determine the excretion pattern of TMZ metabolites.

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