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A liquid chromatography–MS-MS turbulent flow on-line extraction method was developed for the determination of trimebutine (TMB) and its main active metabolite N-mono-desmethyltrimebutine (nortrimetubine or nor-TMB) in human plasma. After protein precipitation and internal standard (IS, haloperidol-d4) addition, 50 µL of the supernatant were transferred onto a Cyclone-Turbo-Flow extraction column followed by an Hypersil PFP Gold analytical column. Detection was carried out on a triple quadrupole tandem mass spectrometer using positive electrospray ionization. The transitions used were m/z 388.0 → 343.0, 374.0 → 195.0 and 380.1 → 169.0 for TMB, nor-TMB and IS, respectively. The method was validated over the concentration range of 10–1,000 ng/mL for both compounds. The accuracy evaluated at three concentrations was within 90.0–98.5% and the intra- and interday coefficient of variation’s for the two molecules were <8.7%. The method was applied to a toxicokinetic study of a self-poisoning case with TMB in a 19-old girl. The concentration of TMB decreased from 747 to 77 ng/mL, while nor-TMB decreased from 9,745 to 205 ng/mL after 5 days and the fatal issue. This case confirms the literature underlining the potential toxicity of TMB, which has long time been considered as a harmless molecule.

Introduction

Trimebutine (TMB), 3,4,5-trimethoxybenzoic acid 2-(dimethyamino)-2-phenylbutyl ester (Figure 1), is an antispasmodic compound widely used in Europe for the treatment of bowel disorders and marketed under the name Debridat®. TMB exhibits a high first-pass hepatic metabolism, and undergoes biotransformation via N-demethylation, ester hydrolysis and conjugation of the hydrolysis products (2). The major circulating compound from TMB metabolism is nortrimetubine (nor-TMB) (Figure 1) which reached a much higher plasma concentration than TMB itself 2 h after oral administration (3). Pharmacokinetic parameters of both compounds are given in Table 1 (3–5).

The mechanism of action of TMB involves weak and non-specific peripheral µ,κ and δ opioid receptor stimulation, sodium channel blockade and local anesthetic properties. Nor-TMB has shown more potent effects than TMB on sodium channel blockade (4, 5).

Oral acute toxicity of TMB is low. However, an anticholinergic effect of TMB can appear in overdose (6). The toxicity of TMB has been attributed to its depressant effect on the electrical activities of the heart by inhibition of both sodium fast channels and calcium slow channels (7), with a low pro-arrhythmic potential (8). But this cardiotoxicity has only been demonstrated in animals with concentrations 100 times higher than those found in clinical practice. Toxicity has been attributed by others to its respiratory depressant effect rather than to its cardiovascular effect (9). TMB poisoning in humans has been reported in few cases. Symptoms included seizures, bradycardia, ventricular tachycardia and arrhythmia (6, 10).

Many analytical methods using liquid chromatography (LC) coupled with either single-wavelength UV, diode-array detection (DAD) (3, 11–13), tandem mass spectrometry (14, 15) or using capillary zone electrophoresis (16) have been previously published for the determination of TMB and nor-TMB in human plasma. No LC–MS-MS method coupled with an on-line extraction for human specimens has been described. A new analytical method using only 200 µL of sample is described, for the identification and quantification of both TMB and nor-TMB. A fatal poisoning case with a toxicokinetic exploration over 5 days following ingestion of toxic amount of TMB is reported here to emphasize the importance of toxicological analysis in the diagnosis and monitoring of acute poisoning by a drug traditionally considered as harmless.

Experimental

Chemicals and reagents

TMB C23H29NO5, monoisotopic mass 387.47 g/mol and nor-TMB C23H28NO4, monoisotopic mass 373.44 g/mol were purchased from Sigma-Aldrich, St-Quentin-Fallavier, France. The internal standard (IS), haloperidol-d4 was obtained from Cerilliant, Sigma-Aldrich. HPLC-grade acetonitrile, ammonium formate and formic acid were supplied by Sigma-Aldrich. HPLC-grade methanol was from Prolabo, VWR International, Fontenay-sous-Bois, France. Ultra-pure water was obtained by reverse osmosis using a Direct-Q UV3 apparatus (Millipore, Molsheim, France). All other chemicals were of analytical grade.

Working solutions, calibration standard and quality controls

Stock solutions of TMB and nor-TMB (1 g/L) were prepared in methanol. The IS solution (haloperidol-d4, 100 mg/L) was prepared ready to use. Working solutions of TMB and nor-TMB for use as calibration standards (CS) were prepared at three concentration levels (0.1, 1 and 10 mg/L) by dilution of the stock solution with methanol. Another 1 g/L stock solution was prepared for quality control (QC) at the same concentration by dilution in methanol. A working solution of the IS (1 mg/L) was obtained by dilution of the stock solution with methanol.
CS and QC solutions were stored at were obtained from a local blood bank (EFS, Versailles, France). 20 m containing 200 to each of 1.5 mL Eppendorf tubes (Dutscher, Brumath, France) Fifty microliters of the IS working solution (1 mg Sample preparation concentrations of 35, 350.0 and 700.0 1,000 g/mL were added to each of 1.5 mL Eppendorf tubes (Dutscher, Brumath, France) containing 200 μL plasma. Proteins were precipitated using 20 μL ZnSO₄ solution (1 mol/L). After vortex mixing and centrifugation (14,000 × g, 10 min), 50 μL of supernatant were transferred into injection vials (12 × 32 mm, double rings, Interchim, Montluçon, France) for analysis.

**Turboflow LC–MS–MS system and conditions**

The TurboFlow device consists of a Cohesive Aria TLX-1 System (version 1.1.1, Thermo Fisher Scientific, Les Ulis, France) equipped with a multiple column module, a quaternary loading pump, a binary eluting pump and a CTC PAL 2.2.0 autosampler. For loading pumps, system eluents used were (A) formic acid 0.1% (C) 10 mmol/L ammonium acetate adjusted to pH 9.0 with ammoniac (D) acetonitrile. For the eluting pump, system eluents used were (A) 2 mmol/L ammonium formate/formic acid 0.1% and (B) acetonitrile. The entire experiment was controlled by Aria operating software 1.1.1. The gradient elution and valve-switching profile of the TurboFlow are summarized in Table II. The total analysis time was 7 min, including column re-equilibration. Compounds were detected by a TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) source. Nitrogen (N2MID350 nitrogen generator; Parker Hannifin France, Contamine-sur-Avre, France) was used as sheath and auxiliary gas at a pressure of 40 and 5 AU, respectively. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. The ESI source was set in positive ionization mode, and an ionspray potential of +4.0 kV was applied. The scan time was 0.03 ms. Capillary temperature was set at 300°C. The system was tuned by using a continuous 3 μL/min infusion of TMB and nor-TMB (5 mg/L in mobile phase). Data were collected in multiple reaction monitoring mode, with two m/z transitions for each compound.

Protonated molecular ions [M+H]+ of TMB (m/z 388.0), nor-TMB (m/z 374.0) and the IS (m/z 380.1) were selected with a mass resolution of ±0.5u (atomic mass unit) and fragmented with different collision energy to obtain two product ions for each parent drug (Table III).

Chromatographic data acquisition was performed using Xcalibur software (version 2.0.7, Thermo Fisher Scientific). Post-analysis processing was carried out using LC Quan software (version 2.5.6, Thermo Fisher Scientific).

**Method validation procedure**

**Selectivity, carry over**

To investigate whether endogenous matrix constituents interfered with the assay, drug-free blank samples and samples spiked at the lower limit of quantification (LLOQ) were analyzed in accordance with the described procedure. assay selectivity was defined by evidence of non-interference at retention times and ion channels identical with those for TMB, nor-TMB and the IS in the blank samples. A blank sample was also analyzed immediately after the highest CS in each run to monitor carry over of the three molecules.

**Linearity**

Calibration curves included a zero sample and seven CS over the concentration range 10 ng/mL (LLOQ) to 1,000 ng/mL (upper limit of quantification, ULOQ). Six calibration curves were obtained over a 10-day period. Quantification was achieved by plotting the peak area ratios of TMB and nor-TMB to the IS. Back-calculated concentrations of the CS had to be within 85–115% of the specified concentrations.

**Lower limit of detection and quantification**

The LLOD was defined as the lowest concentration for which the full MS–MS spectrum could be identified with a signal-to-noise ratio >3.

The LLOQ was defined as the lowest concentration for which accuracy was between 80 and 120% and precision with a

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**Table I**

Pharmacokinetic Characteristics of TMB and Nor-TMB

<table>
<thead>
<tr>
<th>Oral dose (mg)</th>
<th>TMB</th>
<th>Nor-TMB</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cₘₐₓ (ng/mL)</td>
<td>Tₘₐₓ (h)</td>
<td>Half-life (h)</td>
</tr>
<tr>
<td>100</td>
<td>&lt;20</td>
<td>– –</td>
<td>540</td>
</tr>
<tr>
<td>n = 24 200</td>
<td>– –</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>n = 24 300</td>
<td>200</td>
<td>2 –</td>
<td>2,500</td>
</tr>
<tr>
<td>n = 6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Calibration curves were prepared by spiking blank plasma with appropriate volumes of the previously mentioned working solutions to produce CS containing 10, 25, 50, 100, 250, 500 and 1,000 μg/L. QC samples were also prepared in blank plasma at concentrations of 35, 350.0 and 700.0 μg/L.

Blank human plasma samples collected on acid citric dextrose were obtained from a local blood bank (EFS, Versailles, France). CS and QC solutions were stored at −20°C.

**Sample preparation**

Fifty microliters of the IS working solution (1 mg/L) were added to each of 1.5 mL Eppendorf tubes (Dutscher, Brumath, France) containing 200 μL plasma. Proteins were precipitated using 20 μL ZnSO₄ solution (1 mol/L). After vortex mixing and centrifugation (14,000 × g, 10 min), 50 μL of supernatant were transferred into injection vials (12 × 32 mm, double rings, Interchim, Montluçon, France) for analysis.

![Figure 1. Structure of TMB (R = CH₃) and nor-TMB (R = H).](image-url)
Accuracy and precision
The accuracy (measured value/nominal value × 100) and precision (CV) of the method were carried out over 3 days. Each day, one calibration curve and six determinations of each QC level were analyzed. The values obtained were analyzed using analysis of variance, which separated the intra- and inter-assay standard deviation and consequently the corresponding CV. The intra-assay CV took into account the variability of the six replicates each day for 3 days and the inter-assay CV the variability of the days of analysis.

Matrix effect and overall method recovery
To investigate analyte recovery, aqueous solutions containing TMB and nor-TMB at 350 ng/mL were prepared with the IS. After the sample-preparation step, prepared solutions were

1. Subjected to the complete TurboFlow procedure. 2. Analyzed with the TurboFlow system bypassed (i.e., injection directly on to the analytical column). The mean peak areas for each compounds subjected to the complete TurboFlow procedure were compared with those obtained from injection on to the analytical column only, with the latter assumed to represent 100% recovery. Overall method recovery of the IS had to be ± 15% of TMB and nor-TMB recoveries. Another mix solution of the three molecules at the same concentration were prepared in six different blank plasma. To evaluate the matrix effect, plasma and aqueous samples were both subjected to the complete TurboFlow procedure. The mean peak areas of each analytes from the different blank plasma were compared with those observed with the aqueous solutions.

Results and discussion
As deuterated-TMB compound was not available, haloperidol-d₄ was chosen as an IS due to the similar physicochemical properties, chemical structure and the same extraction behavior than the relevant molecules, and seems a good candidate for the assay. Under the optimized conditions, TMB, nor-TMB and IS were eluted with retention times of 4.79, 4.70 and 4.88 min, respectively (Figure 2). Atropine, scopolamine, psychotropic drugs as antidepressant, anxiolytics (all benzodiazepine for example), hypnotics (zolpidem and zopiclone), neuroleptics and anticonvulsivants were tested and no interference was observed.

Method validation
Selectivity and carry over
No interferences from the constituents of drug-free human plasma samples were observed at the retention times and ion channels of TMB, nor-TMB or the IS. When a blank sample was analyzed immediately after the highest CS, mean carry over was < 0.1% for the three compounds (data not shown).

Linearity, limit of quantification, limit of detection and dilution effect
Quantification was achieved by linear regression analysis, which was the best fitting model as determined by bias analysis. The seven-point calibration curve was linear in the concentration range 10–1,000 ng/mL with 1/x weighting factor; correlation coefficients ranged from 0.998 to 0.999 for TMB and from 0.996 to 0.999 for nor-TMB. The LLOQ was 10 ng/mL for both TMB and nor-TMB with CV of 5.7 and 8.2%, respectively. The ULOQ was 1,000 ng/mL. The 1/5 and 1/10 dilutions of the high QC samples were tested and back-calculated concentrations of diluted QC samples were accurate compared with the specified concentrations. The LOD was 1 ng/mL with a signal-to-noise ratio > 3.

Precision and accuracy
The accuracy evaluated at three concentrations was within 90.0–98.5% of the specified concentrations and the intra- and interday CV’s for the two molecules were all <8.7% (Table IV).
Matrix effect and overall method recovery
Matrix effect was of 145% for TMB and of 171% for nor-TMB with the same overall recovery of 72%. Matrix effect for the IS was of 63% and overall recovery was of 75%.

These results showed a different matrix effect between the couple TMB/nor-TMB and the IS. However, haloperidol-d4 remained the favorite IS due to its complete extractive behavior similarity with the two compounds.

Application: case report
A 19-year-old girl who voluntarily ingested numerous tablets of aspirin, acetaminophen and TMB (~120 tablets of 200 mg TMB) was referred to the Emergency Medical Service by her stepfather following convulsions and she was found to be in cardio respiratory arrest. Tracheal intubation, cardiac massage and repetitive bolus of adrenaline following several bradycardia and ventricular fibrillation episodes allowed the establishment
of a spontaneous cardiac activity. The patient was then transferred to a Medical Intensive Care Unit at the regional hospital. On admission, examination showed a bilateral pyramidal syndrome with reactive miosis and aspiration pneumonia. Toxicological analysis was requested. Blood and urine immunoassay screening (narcotics, barbiturates, benzodiazepines, carbamates, acetaminophen and tricyclic antidepressants) were carried out. Non-specific screening was performed on blood and urine by HPLC–DAD, GC–MS and LC–MS–MS after liquid–liquid extraction in acidic and basic conditions. Alcohol was determined by GC/FID in both blood and urine. The present method for identification and quantification of TMB and Nor-TMB was applied to a series of plasma samples obtained after centrifugation of blood collected on heparinized tubes at different times.

In the first specimen collected 5 h after ingestion, alcohol and drugs of abuse were negative. Only acetaminophen and salicylic acid were found, at plasma supra-therapeutic and therapeutic concentrations (56 and 180 mg/L, respectively). Plasma concentrations of TMB and nor-TMB obtained after 5, 34, 55, 85, 103 and 127 h post-ingestion are given in Figure 3. These results showed an important maximum measured concentration for TMB at 747 ng/mL observed 5 h post-ingestion. However, according to the $T_{\text{max}}$ published in the literature, around 2–3 h (3), it is likely that this concentration had been higher prior to the initial time of specimen collection. For nor-TMB, a much higher maximum concentration was observed at 9,745 ng/mL, obtained for the plasma sampled at 34 h. The $T_{\text{max}}$ of nor-TMB was probably between 5 and 34 h since the plasma concentration increased between these two sampling times, in disagreement with literature where $T_{\text{max}}$ of nor-TMB was always reported around 2–3 h (3). This delayed metabolism is probably due to cardiac arrest initially suffered by the victim. These concentrations of TMB and nor-TMB observed in this patient appeared very important, since therapeutic concentrations of these two compounds are evaluated at 150 ng/mL for TMB and 2,500 ng/mL for nor-TMB (3). Moreover, plasma elimination half-life of TMB and nor-TMB, being usually around 2–3 h for TMB and 7 h for nor-TMB (3), was prolonged in our patient since these two compounds remained detectable 5 days following ingestion, without important renal failure or hepatic disorder that could explain the decrease in clearance of TMB and its metabolite. Using log-scale graph, we could estimate the half-life of TMB and nor-TMB to be $\sim$30 and 40 h, respectively.

Since 2000, five case reports featuring six cases of TMB poisoning have been published (17–21) and are presented in Table V. In our case, nor-TMB blood concentration found 34 h after ingestion was $\sim$10,000 ng/mL. None of the cited papers have reported such a level of nor-TMB. Nor-TMB has shown more potent effects than TMB specially on sodium channel blockade and glutamate inhibition (4, 5). This finding could explain cardio- and neurotoxicity of TMB described in earlier publications, and substantiates the origin of cardiorespiratory arrest as being due to a possible membrane stabilizing effect of TMB in our case report. Our patient died due to neurological complications worsened by the initial hypoxic ischemia.

![Figure 3. Toxicokinetic of TMB (filled circle) and nor-TMB (filled square) from the case report.](image-url)

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Precision and Accuracy for Plasma QC Samples of TMB and Nor-TMB</th>
</tr>
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<tbody>
<tr>
<td>QC concentration (ng/mL)</td>
<td>35</td>
</tr>
<tr>
<td>TMB</td>
<td></td>
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<tr>
<td>Precision intraday (%)</td>
<td>8.5</td>
</tr>
<tr>
<td>Precision interday (%)</td>
<td>4.9</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>97.1</td>
</tr>
<tr>
<td>Nor-TMB</td>
<td></td>
</tr>
<tr>
<td>Precision intraday (%)</td>
<td>9.2</td>
</tr>
<tr>
<td>Precision interday (%)</td>
<td>4.7</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>98.5</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table V</th>
<th>Published Cases of TMB Poisoning with TMB and/or Nor-TMB Quantitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases ($n=6$)</td>
<td>Assumed ingested dose (mg)</td>
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<tr>
<td>---</td>
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</tr>
<tr>
<td>Sex</td>
<td>Age</td>
</tr>
<tr>
<td>M/F</td>
<td>(years)</td>
</tr>
<tr>
<td>F</td>
<td>19</td>
</tr>
<tr>
<td>F</td>
<td>?</td>
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<tr>
<td>F</td>
<td>18</td>
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<tr>
<td>H</td>
<td>57</td>
</tr>
<tr>
<td>F</td>
<td>14</td>
</tr>
<tr>
<td>F</td>
<td>17</td>
</tr>
</tbody>
</table>

[M], male; [F], female; (?), not mentioned; (h), heart; (p), peripheral; (u), unknown; (NP), not performed.
Conclusion

We developed a novel and reliable automated LC–MS-MS method using turbulent flow on-line extraction technology for simultaneous determination of TMB and nor-TMB and demonstrated its utility in the first toxicokinetic study in a fatal case. Our case report confirms that the literature underlining the potential severity of TMB intoxication and the difficulty of interpreting toxicological results due to a lack of reference values and toxic ranges of this compound, which has long time been considered as harmless molecule.

References