Simultaneous Determination of Tramadol and Its Metabolite in Human Urine by the Gas Chromatography–Mass Spectrometry Method

Bilal Yilmaz1* and Ali Fuat Erdem2

1Department of Analytical Chemistry, Faculty of Pharmacy, Ataturk University, Erzurum 25240, Turkey, and 2Department of Anesthesiology and Reanimation, Faculty of Medicine, Sakarya University, Sakarya 54187, Turkey

*Author to whom correspondence should be addressed. Email: yilmazb@atauni.edu.tr

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A sensitive and efficient method was developed for determination of tramadol and its metabolite (O-desmethyltramadol) in human urine by gas chromatography–mass spectrometry. Tramadol, O-desmethyltramadol and medazepam (internal standard) were extracted from human urine with a mixture of ethylacetate and diethylether mixture (1 : 1, v/v) at basic pH with liquid–liquid extraction. The calibration curves were linear (r = 0.99) over tramadol and O-desmethyltramadol concentrations ranging from 10 to 200 ng/mL and 7.5 to 300 ng/mL, respectively. The method had an accuracy of >95% and intra- and interday precision (relative standard deviation %) of <4.93 and <4.62% for tramadol and O-desmethyltramadol, respectively. The extraction recoveries were found to be 94.1 ± 2.91 and 96.3 ± 3.46% for tramadol and O-desmethyltramadol, respectively. The limit of quantification using 0.5 mL human urine was 10 ng/mL for tramadol and 7.5 ng/mL for O-desmethyltramadol. After oral administration of 100 mg of tramadol hydrochloride to a patient, the urinary excretion was monitored during 24 h. About 15% of the dose was excreted as unchanged tramadol.

Introduction

Tramadol (Figure 1), (±)-trans-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl) cyclohexanol, is an analgesic used in treatment of mild pain (1). The plasma concentration in therapeutic terms has a domain between 100 and 300 ng/mL, being almost completely and quickly absorbed through a metabolism giving different metabolites due to the action of cytochrome P450 isozyme CYP2D6 (2–6). The bio-availability ranges from 65 to 70% because of the early stage of metabolism, knowing also that 6% of population has a slow CPY2D6 activity giving a slightly reduced analgesic effect. Tramadol is transformed to O- and N-desmethyl compounds (7–9).

Several analytical methods for determination of tramadol and its metabolites were used, starting from gas chromatography (GC) with nitrogen selective or mass spectrometry (MS) detection (10–12), but recently the new separation methods were introduced, capillary electrophoresis (13) and high-performance liquid chromatography (HPLC) methods with electrochemical (14), MS (15–18) or fluorescence detection (19–24).

El-Sayed et al. (10) have developed a GC–MS method for the determination of tramadol and O-desmethyltramadol in human urine with positive electron impact ionization following β-glucuronidase hydrolysis and liquid–liquid extraction. The proposed method has a total run time of 12.6 min. A linear calibration curve was obtained over the range 10–1,000 ng/mL. Intra-assay precision was within 1.29–6.48% and inter-assay precision was within 1.28–6.84% for tramadol and O-desmethyltramadol. Intra-assay accuracy was within 91.79–106.89% for tramadol and O-desmethyltramadol. This method is also the most comprehensive method which can extract tramadol in a single extraction procedure. Our recovery is better than those of the studies reported by El-Sayed et al. Our method has a total run time 8.0 min which is faster than previously published GC–MS methods for tramadol and its metabolites (10).

Ghasemi (11) has developed a GC–MS method for the determination of tramadol in different biological samples using solvent bar microextraction. The detection limit was 0.02 μg/L with 4.5% relative standard deviation (RSD). Tramadol was extracted from plasma with a solid-phase extraction (SPE) procedure. This method is also the most comprehensive method, which can extract tramadol in a single extraction procedure. The method has a total run time of 15 min. In our study, the retention time of tramadol was much shorter than that studied by Ghasemi (11).

The UV detection from biological samples is not suitable because of low sensitivity and selectivity, although there is a benzene ring present in tramadol and its metabolite molecules (25–30). For the moment, only two types of detectors have reached low quantification levels: MS and fluorescence. By using any of them, the lowest level for tramadol quantification was ~1.0 mg/mL (12, 19, 21), meaning that the lowest limit of detection was ~0.2–0.5 ng/mL (12, 19).

Ebrahimzadeh et al. (31) have developed multivariate optimization of surfactant-assisted directly suspended droplet microextraction combined with GC for the preconcentration and determination of tramadol in biological samples. In this technique, a free microdroplet of solvent is transferred to the surface of an immiscible aqueous sample containing Triton X-100 and tramadol while being agitated by a stirring bar placed on the bottom of the sample vial. After the predetermined time, the microdroplet of solvent is withdrawn by a syringe and analyzed.

Pinho et al. (32) have developed simultaneous quantification of tramadol and O-desmethyltramadol in hair samples by GC–MS. In this study, a methodology aimed at the simultaneous quantification of tramadol and metabolite in human hair was developed and validated. After decontamination of hair samples (60 mg), tramadol and metabolite were extracted with methanol in an ultrasonic bath (~5 h). Purification was performed by SPE using mixed-mode extraction cartridges. Subsequently to derivatization, analysis was performed by GC–MS.

Several methods have been reported for determination of tramadol, including the GC–MS method, human plasma, saliva, hair and biological materials. But, on extensive survey of the literature, no
method is reported till date for determination of tramadol and metabolite by the GC–MS method without derivatization in patient human urine. Therefore, the aim of our study was to develop a specific, sensitive, precise and accurate GC–MS method for analysis of tramadol and its metabolite in patient human urine. The proposed method was fully validated in respect to limits of detection (LOD) and limits of quantification (LOQ), precision, accuracy, linearity, specificity, stability and extraction recovery parameters according to International Conference on Harmonization (ICH) guidelines (33).

The advantages of the present method include a simple and single-step extraction procedure using inexpensive chemicals and short run time. Besides, this method was used to assay the tramadol and O-desmethyltramadol in urine samples obtained from a patient. At the same time, this method was used to a pharmacokinetic study after therapeutic doses of tramadol and O-desmethyltramadol.

Experimental

Chemicals and reagents

Tramadol hydrochloride and O-desmethyltramadol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Medazepam was obtained from Criminal Police Laboratory (Erzurum, Turkey). Sodium carbonate, ethylacetate, hexane, diethylether, dichloromethane, acetonitrile, butanol and chloroform were purchased from Sigma-Aldrich. Contramal tablet drug (100 mg tramadol hydrochloride) was obtained from the pharmacy (Erzurum, Turkey).

Apparatus and analytical conditions

Chromatographic analysis was carried out on an Agilent 6890N GC system equipped with 5973 series mass selective detector, 7673 series autosampler and chemstation (Agilent Technologies, Palo Alto, CA, USA). A HP-5 MS column with 0.25 μm film thickness (30 m × 0.25 mm ID) was used for separation. Splitless injection was used and the carrier gas was helium at a flow rate of 1 mL/min. The injector and detector temperatures were 250°C. The temperature programs of the GC oven were as follows: initial temperature 70°C, held for 1 min, increased to 250°C at 35°C/min held for 1 min and finally to 290°C at 20°C/min with a final hold of 1 min. The MS detector parameters were transfer line temperature 290°C, solvent delay 3 min and electron energy 70 eV. The MS was run in scan mode (m/z 40–500) for qualitative analysis and selected ion monitoring mode for quantitative analysis [(m/z 58 for tramadol and O-desmethyltramadol, and m/z 207 for internal standard (IS)] (Figure 2).

Preparation of stock and standard solutions

Stock solutions of tramadol and O-desmethyltramadol were prepared by dissolving the accurately weighed reference compounds in methanol to give a final concentration of 10 μg/mL of both. The solutions were then serially diluted with methanol to achieve standard working solutions at concentrations of 10, 25, 50, 100, 150, 175 and 200 ng/mL and 7.5, 15, 25, 50, 100, 200 and 300 ng/mL for tramadol and O-desmethyltramadol, respectively. Stock solution of IS was prepared in methanol at the concentration of 5 μg/mL. All the solutions were stored at 4°C and were brought to room temperature before use. The quality control (QC) solutions were prepared by adding aliquots of standard working solution of tramadol and O-desmethyltramadol to final concentrations of 12.5, 75 and 175 ng/mL and 7.5, 75 and 250 ng/mL containing 0.1 mL IS (500 ng/mL).

Extraction procedure

The liquid–liquid extraction method was used in this study. Several solvents (ethylacetate, diethylether, dichloromethane, acetonitrile, butanol and chloroform) were tested for the extraction. Finally, the ethylacetate and diethylether mixture (1:1, v/v) proved to be the most efficient in extracting tramadol and O-desmethyltramadol from human urine. A 0.5 mL of the urine samples was spiked with 1 mL of tramadol or O-desmethyltramadol, 0.1 mL of IS and 1 mL of 0.1 M sodium carbonate were added. After vortex mixing for 5 s, 4 mL of ethylacetate and diethylether mixture (1:1, v/v) were added. The mixture was vortexed for 30 s and then centrifuged at 3,000 × g for 3 min. The organic layer was transferred into another tube and evaporated to dryness at room temperature under nitrogen gas. The dry residue was dissolved in 100 μL of methanol. The mixture was vigorously shaken and then 1 μL sample was injected into the GC–MS system.

Results

Method development and optimization

The method development for the assay of tramadol and O-desmethyltramadol was based on their chemical properties.
In this study, the capillary column coated with 5% phenyl and 95% dimethylpolysiloxane is a good choice for separation of these analytes because they elute as symmetrical peaks at a wide range of concentrations. Different temperature programs were investigated for GC oven. At the end of this investigation, the best temperature program was selected for a good separation. The splitless injection mode was chosen. Additionally, preliminary precision and linearity studies performed during the development of the method showed that the 1 µL injection volume was reproducible and the peak response was significant at the analytical concentration chosen.

**Validation of the method**

To evaluate the validation of the present method, parameters as selectivity, linearity, precision, accuracy, LOD and LOQ, recovery and stability were investigated according to the ICH validation guidelines (33).

**Selectivity**

The selectivity of the assay was checked by comparing the chromatograms of batches of blank urine with the corresponding spiked urine. Each blank sample was tested for the observation of interference, and no endogenous interferences were encountered (Figure 3a). The retention time of tramadol and O-desmethyltramadol in human urine was ~6.2 and 6.5 min with good peak shape (Figure 3b).

**Linearity**

Calibration curves for the urine assay developed with a peak-area ratio (y) of tramadol and O-desmethyltramadol to IS versus drug concentration were found to be linear over the concentration range 10–200 and 7.5–300 ng/mL using a weighted least squares method. The linear regression equations of the calibration curves of tramadol and O-desmethyltramadol are summarized in Table I. The correlation coefficients were found to be >0.99.

**Precision and accuracy**

The precision of the analytical method was determined by repeatability (intraday) and intermediate precision (interday). Repeatability was evaluated by analyzing spiked blank urine six times per day, at three different concentrations which were urine QC samples. The intermediate precision was evaluated by analyzing the same urine samples once daily for 3 days. The RSD of the predicted concentrations from the regression equation was taken as precision. The accuracy of this analytical method was assessed as the percentage relative error. For all the concentrations studied, intra- and interday RSD values were ≤4.93% and for all concentrations of tramadol and O-desmethyltramadol the relative errors were ≤5.89%. These results are given in Table II.

**LOD and LOQ**

The sensitivity was evaluated by the LOQ, the lowest concentration of the urine spiked with tramadol or O-desmethyltramadol.
in the calibration curve. The LOD was determined as the lowest concentration, which gives a signal-to-noise ratio of 3 for tramadol or O-desmethyltramadol. Under the experimental conditions, the LOQ values were 10 and 7.5 ng/mL for tramadol and O-desmethyltramadol, respectively. Furthermore, the LOD values were 3.0 ng/mL for tramadol and 2.5 ng/mL for O-desmethyltramadol (Table I).

Recovery

For extraction of tramadol and O-desmethyltramadol from human urine, the liquid–liquid extraction technique was tried using ethylacetate, diethylether, dichloromethane, acetonitrile, butanol and chloroform. It was observed that extraction yields were very low. The urine sample was done alkaline by 1 mL of 0.1 M sodium carbonate solution and then tramadol and O-desmethyltramadol were extracted from urine using 4 mL of ethylacetate and diethylether mixture (1 : 1, v/v).

The analytical recovery of tramadol and O-desmethyltramadol from human urine was assessed by direct comparison of concentrations of tramadol and O-desmethyltramadol obtained after the whole extraction procedure by using six replicates at different concentration levels in the calibration graph versus standard tramadol and O-desmethyltramadol solutions. The extraction recoveries of tramadol and O-desmethyltramadol from human urine were between 88 and 104% as summarized in Table III.

Matrix effect

The blank urines used in this study were from three different batches of healthy human urine. If the ratio <85 or >115%, a matrix effect was implied. The relative matrix effect of tramadol and O-desmethyltramadol at three different concentrations was less than ±1.2% (Table IV). The results showed that there was no matrix effect of the analytes observed from the matrix of urine in this study.

Stability

The stability of tramadol and O-desmethyltramadol in human urine was studied under a variety of storage and handling conditions at low (50 ng/mL) and high (300 ng/mL) concentration levels. The short-term temperature stability was assessed by analyzing three aliquots of each of the low and high concentration samples that were thawed at room temperature and kept at this temperature for 8 h. Freezer–thaw stability (−20°C in urine) was checked through three cycles. Three aliquots at each of the low and high concentrations were stored at −20°C for 24 h and thawed unassisted at room temperature. When completely

Table I

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tramadol (ng/mL)</th>
<th>O-Desmethyltramadol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>10–200</td>
<td>7.5–300</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$y = 0.025x + 0.0711$</td>
<td>$y = 0.034x + 0.134$</td>
</tr>
<tr>
<td>Standard deviation of slope</td>
<td>$5.2 \times 10^{-2}$</td>
<td>$2.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Standard deviation of intercept</td>
<td>$1.5 \times 10^{-2}$</td>
<td>$2.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>Standard deviation of correlation coefficient</td>
<td>$5.5 \times 10^{-4}$</td>
<td>$4.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>Limit of detection (ng/mL)</td>
<td>3.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Limit of quantification (ng/mL)</td>
<td>10</td>
<td>7.5</td>
</tr>
</tbody>
</table>

*Based on six calibration curves, y is the peak-area ratio and x is the concentration (ng/mL).

Table II

<table>
<thead>
<tr>
<th>Added (ng/mL)</th>
<th>Intraday</th>
<th>Interday</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found ± SD</td>
<td>Precision</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% RSDa</td>
</tr>
<tr>
<td>Tramadol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>11.92 ± 0.223</td>
<td>1.87</td>
</tr>
<tr>
<td>75</td>
<td>71.47 ± 1.251</td>
<td>1.35</td>
</tr>
<tr>
<td>175</td>
<td>189.54 ± 1.635</td>
<td>0.97</td>
</tr>
<tr>
<td>O-Desmethyltramadol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>7.41 ± 0.342</td>
<td>4.62</td>
</tr>
<tr>
<td>75</td>
<td>70.58 ± 1.532</td>
<td>2.17</td>
</tr>
<tr>
<td>250</td>
<td>242.62 ± 3.164</td>
<td>1.30</td>
</tr>
</tbody>
</table>

SD, standard deviation of six replicate determinations; RSD, relative standard deviation.

*Average of six replicate determinations.

Accuracy (% relative error) = (found – added)/added × 100.
the thawed, the samples were refrozen for 24 h under the same con-
ditions. The freeze–thaw cycles were repeated three times and
then analyzed on the third cycle. The long-term stability was
determined by analyzing three aliquots of each of the low and high
concentrations stored at $-20^\circ$C for 1 week. The results of the
stability studies are given in Table V and no significant degrada-
tion of tramadol and O-desmethyltramadol was observed under
the tested conditions.

Application of the method
Prior to the study, the clinical protocol was approved by the
Ethics Committee of Faculty of Medicine, Ataturk University.

Table III
Recovery of Tramadol and O-Desmethyltramadol in Human Urine

<table>
<thead>
<tr>
<th>Added (ng/mL)</th>
<th>Found (mean ± SD)</th>
<th>% Recovery</th>
<th>% RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tramadol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8.8 ± 0.44</td>
<td>88</td>
<td>5.0</td>
</tr>
<tr>
<td>25</td>
<td>24 ± 0.38</td>
<td>96</td>
<td>4.1</td>
</tr>
<tr>
<td>50</td>
<td>47 ± 2.41</td>
<td>94</td>
<td>5.1</td>
</tr>
<tr>
<td>100</td>
<td>97 ± 3.84</td>
<td>97</td>
<td>3.9</td>
</tr>
<tr>
<td>150</td>
<td>141 ± 5.23</td>
<td>94</td>
<td>3.7</td>
</tr>
<tr>
<td>175</td>
<td>162 ± 6.17</td>
<td>93</td>
<td>3.8</td>
</tr>
<tr>
<td>200</td>
<td>183 ± 6.42</td>
<td>97</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table IV
Matrix Effect Evaluation of Tramadol and O-Desmethyltramadol and IS in Human Urine (n = 3)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration level (ng/mL)</th>
<th>A (mean ± SD)</th>
<th>B (mean ± SD)</th>
<th>% Matrix effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tramadol</td>
<td>50</td>
<td>47 ± 2.86</td>
<td>103 ± 8.45</td>
<td>1.06</td>
</tr>
<tr>
<td>100</td>
<td>97 ± 6.98</td>
<td>103 ± 8.45</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>202 ± 8.67</td>
<td>198 ± 9.73</td>
<td>-0.98</td>
<td></td>
</tr>
<tr>
<td>0-Desmethyltramadol</td>
<td>50</td>
<td>46 ± 3.74</td>
<td>54 ± 2.87</td>
<td>1.17</td>
</tr>
<tr>
<td>150</td>
<td>136 ± 8.28</td>
<td>145 ± 6.62</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>232 ± 9.14</td>
<td>253 ± 8.64</td>
<td>1.09</td>
<td></td>
</tr>
</tbody>
</table>

Table V
Stability of Tramadol and O-Desmethyltramadol in Human Urine Under Various Storage Conditions (n = 3)

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Concentration (ng/mL)</th>
<th>Calculated concentration (ng/mL)</th>
<th>% RSD</th>
<th>% Relative error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tramadol</td>
<td>Room temperature for 8 h</td>
<td>50</td>
<td>48</td>
<td>2.8</td>
</tr>
<tr>
<td>75</td>
<td>143</td>
<td>4.4</td>
<td>-4.7</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>141</td>
<td>3.1</td>
<td>-2.9</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>144</td>
<td>5.9</td>
<td>-6.0</td>
<td></td>
</tr>
<tr>
<td>1 week at $-20^\circ$C</td>
<td>50</td>
<td>51</td>
<td>3.4</td>
<td>2.0</td>
</tr>
<tr>
<td>100</td>
<td>144</td>
<td>5.8</td>
<td>-4.0</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>145</td>
<td>5.9</td>
<td>-3.3</td>
<td></td>
</tr>
<tr>
<td>0-Desmethyltramadol</td>
<td>Room temperature for 8 h</td>
<td>150</td>
<td>141</td>
<td>7.4</td>
</tr>
<tr>
<td>300</td>
<td>316</td>
<td>6.5</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>1 week at $-20^\circ$C</td>
<td>150</td>
<td>141</td>
<td>5.6</td>
<td>-6.0</td>
</tr>
<tr>
<td>200</td>
<td>319</td>
<td>6.7</td>
<td>6.3</td>
<td></td>
</tr>
</tbody>
</table>

Dilution integrity
The dilution integrity experiment was performed with an aim to
validate the dilution test to be carried out on higher analyte con-
centration above the upper limit of quantification, which may be
counted to be encountered during real subject sample analysis. The precision
and accuracy values for one-fifth and one-tenth dilution ranged from 3.29 to 7.28% and 98.2 to 102.7 for tramadol and
O-desmethyltramadol.

Discussion
Today, GC–MS is a powerful technique for highly specific and
quantitative measurements of low levels of analytes in biological
samples. As compared with HPLC, high-resolution capillary GC
has been less frequently used (34, 35). However, it has inherently
high resolving power and high sensitivity with excellent precision
and accuracy allowed simultaneous detection of tramadol and
O-desmethyltramadol. In addition, the detection limits were lowered to ng levels by GC combined with MS.

The specificity of the method was verified by investigating the
peak interference from the endogenous urine substances.
Representative chromatograms obtained after 12 h adminis-
tration of the drug are shown in Figure 3c. Amounts of excreted
tramadol and O-desmethyltramadol compounds were obtained
by multiplication of the calculated concentration by the mea-
sured excreted urine volume during the time interval (Figure 4).
(0.5 mL) were thawed at a temperature of 25°C (room temperature) and 0.75 mL water and IS (0.5 mL at 1.0 μg/mL concentration) were added to the urine. The mixture was vortexed and transferred to the SPE cartridge. Then, the cartridge was washed with a mixture of acetonitrile–water (2 mL, 15/85) and 3 mL hexane. The eluate was collected from with 3 mL acetonitrile. The eluate was evaporated at 50°C under nitrogen. The residue was dissolved in methanol and 1 μL volume was injected into GC–MS system. The extraction recoveries of tramadol and O-desmethyltramadol from human urine were between 53.2 and 61.8%. Therefore, the liquid–liquid extraction method was used in this study.

Figure 3 shows the cumulative urinary excretion curve for tramadol for a single dose of Contramal tablet (100 mg tramadol hydrochloride). Although our results were obtained from only one subject, our results are in agreement with the literature (13).

Conclusion
A novel, simple, specific and sensitive GC–MS method was described for analysis of tramadol and O-desmethyltramadol in human urine. Furthermore, the method has good linearity, precision, accuracy and sensitivity according to the results obtained from validation data. Additional advantages of this method include small sample volume (0.5 mL), good extraction recovery from urine and a readily available IS. In addition, the extraction procedure in this study was simple. No significant interferences and matrix effect caused by endogenous compounds were observed. Therefore, the method can be very useful and an alternate to performing pharmacokinetic studies in determination of tramadol and O-desmethyltramadol for clinical use.

Acknowledgments
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