

Development and Validation of a Spectrophotometric Method for the Determination of Tramadol in Human Urine Using Liquid-Liquid Extraction and Ion Pair Formation

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Abstract Tramadol is a widely used analgesic and one of the most common drug abuses in Egypt and Middle East. A simple, sensitive and rapid method for the analysis of tramadol in human urine was developed and validated. The analyte was isolated from basified urine using a single liquid-liquid extraction then analyzed by spectrophotometry after ion-pair formation. The calibration curves were linear in the range of 10 -50 µg/mL, the limit of quantitation and the limit of detection were calculated. The method was also valid to determine tramadol at higher concentrations (250 µg/mL) after dilution. The resulting method demonstrated intra and inter-day precision within 6.3 and 9.5%, respectively, and accuracy within ± 9.9 and $\pm 3.8\%$, respectively. There were no interferences from common analgesic, caffeine and nicotine.

Keywords Tramadol, Liquid-Liquid Extraction, Human Urine

1. Introduction

Tramadol [(±) trans-2-(dimethylaminomethyl)-1-(3-methoxy-phenyl)-cyclohexanol hydrochloride][1] (Figure.1) is a centrally acting opioid analgesic. It is structurally related to codeine and morphine, consists of two enantiomers. Both enantiomers and their metabolites contribute to analgesic activity by different mechanisms. (+)-Tramadol and its metabolite (+)-Odesmethyl-tramadol (M1) are weak agonists of the μ opioid receptor. (+)-Tramadol inhibits serotonin reuptake and (-)-tramadol inhibits norepinephrine reuptake[2]. Tramadol is rapidly absorbed after oral administration with a bioavailability of 65-70%. The peak plasma concentration is reached in 1-3 hours after oral administration of capsules; the therapeutic plasma concentration is in the range of 100-300 ng/mL and approximately 10-30% of the parent drug is excreted unchanged in the urine[2-4].

Various methods have been reported for the determination of tramadol in bulk, pharmaceutical preparations, biological fluids and hair including spectrophotometry[5-11], high performance liquid chromatography (HPLC) with different detectors[3],[4],[12-17], gas chromatography (GC) with flame ionization detector[18], gas chromatography-mass

spectrometry (GC-MS)[19], capillary electrophoresis with electrochemiluminescence detection[20-21], voltammetry [22], [23], potentiometry[24-26] and conductometry[24].

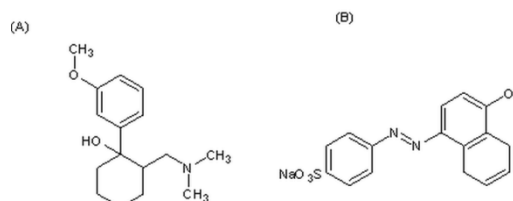


Figure 1. Chemical structures of (A) Tramadol and (B) Tropaeolin OOO

Tramadol is a widely prescribed drug for moderate to severe, acute and chronic pain[2]. Many studies have been indicated that tramadol may induce both classical opioid and atypical withdrawal symptoms[27]. A fatal intoxication in an adult with tramadol alone has been reported, the highest tramadol concentrations were measured in urine and bile and the peripheral blood concentration was at least 30 times higher than the therapeutic range. It was concluded that a high dose of tramadol may lead to death even in the absence of interacting drugs[28]. A tramadol drug abuse phenomenon has been demonstrated in the Egyptian community in the last few years. This phenomenon is not only in Egypt but also in other countries in the Middle East due to its lower price, illegal transactions and availability without prescription. The Egyptian government already added tramadol to the narcotics list. It was reported that different forms of violence, fatalities and accidents in the Egyptian community are closely related to tramadol abuse

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and a tramadol drug screening is significantly required to be added to all toxicological screening tests applied for violence, traffic accidents and also for people applied for drivers testing[29]. Spectrophotometric methods are simple, reproducible and fast. Ultraviolet-visible spectrophotometer is a relatively cheap apparatus and available in most research, clinical and toxicology laboratories. Using of urine samples for detection and quantitation of drugs has many advantages such as usefulness especially in case of pediatric and geriatric patients, noninvasive samples, large volumes and large number of samples can be easily collected[4]. To the best of our knowledge there is no validated spectrophotometric method for the determination of tramadol in human urine. The aim of this work is to develop and validate a simple, rapid and quantitative spectrophotometric method that can be used for routine analysis and screening of tramadol in human urine. The method will be fully validated according to the FDA guidelines[30].

2. Experiment

2.1. Apparatus

A Shimadzu UV and visible recording spectrophotometer (UV 260) with two 10-20 mm matched quartz cells were employed for all absorbance measurements.

2.2. Reagents

All materials and reagent used were of analytical grade, solvents were of spectroscopic grade and double distilled water was used. Tramadol hydrochloride was obtained from Minapharm, Cairo, Egypt under license of Grunenthal, Germany. Dichloromethane (DCM) was purchased from Prolabo, Paris, France. Sodium hydroxide, hydrochloric acid, anhydrous sodium acetate and glacial acetic acid were obtained from BDH, Middle East, LLC. 0.1% aqueous solution of tropeolin OOO (Prolabo, Paris, France), and Acetate Buffer pH 3.7[26] were prepared in double distilled water. Human urine samples were collected freshly from healthy adult volunteers.

2.3. Preparation of Stock Solutions

Two separate 1.0 mg/ml stock solutions of tramadol hydrochloride were prepared in water; each solution was prepared by dissolving 10.0 mg of tramadol hydrochloride in double distilled water in a 10 ml volumetric flask. One stock solution was used for preparation of calibration standards and the other one was used for preparation of quality control (QC) samples. All solutions were stored at approximately 4°C.

2.4. Preparation of Standards and Quality Control (QC) Samples

The calibration standards were prepared by adding the appropriate amounts of the stock solution into pooled blank human urine at concentrations of 10, 20, 30, 35, 40, 45 and

50 µg/mL. QC samples (low, mid, high and dilution QC) were prepared at concentrations of 15, 25, 37.5 and 250 µg/mL in pooled blank human urine. All samples were stored at -20°C

2.5. Liquid-Liquid Extraction and Ion Pair Formation

Human urine samples were thawed at room temperature and mixed well. A 3.0 mL aliquot of each sample was placed into a screw cap culture tube, 1.0 mL of 2N sodium hydroxide, was added and mixed briefly, samples were extracted by addition of 5.0 mL of dichloromethane (DCM) followed by rotation for approximately 2 min. After centrifugation at approximately 3000 rpm for 10 min, 4.0 mL of the organic layer was quantitatively transferred to a separating funnel, 1.0 mL of 2N HCl was added and mixed briefly, 1.5 mL of 0.1 % tropeolin OOO w/v reagent was added, followed by 2.0 mL of acetate buffer solution pH (3.7) and mixed well. The absorbance of the resulting color in the organic layer was measured against the reagent blank at 470 nm (Figure. 2).

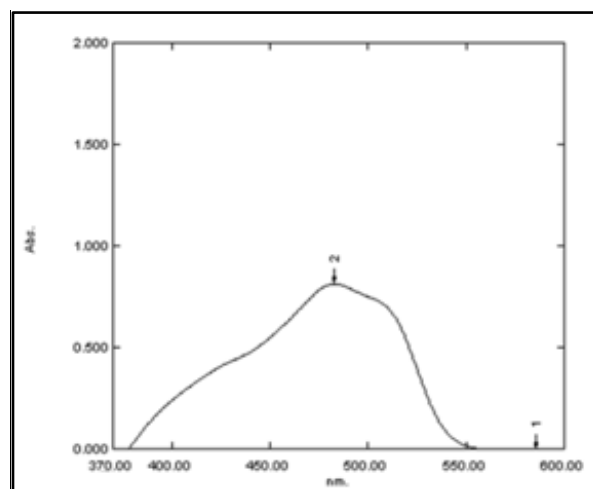


Figure 2. Absorption spectra of the reaction product of 40 µg/mL tramadol with 0.1 % tropeolin OOO w/v, w/v solution

2.6. Validation

The resulting method was validated according to the FDA guidelines[30]

2.6.1. Linearity, LLOQ and LLOD

The urine calibration curve was constructed using seven calibration standards in duplicate over four validation runs. Linearity was established by least squares linear regression analysis of the calibration curve. Absorbance was plotted versus tramadol concentration. The lower limit of quantitation (LOQ) and the lower limit of detection were calculated as $10 \sigma/S$ and $3.3 \sigma/S$, respectively, where σ is the standard deviation of the blank responses ($n=12$) and S is the slope of the calibration curves.[31].

2.6.2. Selectivity

Human urine samples, from six different individuals were extracted and analyzed for interferences at 470 nm

2.6.3. Cross Analyte Interference

Cross analyte interferences from different analgesic (aspirin, ibuprofen, diclofenac sodium, piroxicam and acetaminophen) and from caffeine were evaluated by analysis of 3 aliquots of blank human urine that were fortified individually with each analytes at concentration of 50 µg/mL. Potential interferences from nicotine and its metabolites were evaluated by analyzing urine samples from a smoker individual; average 20 cigarettes per day and three passive smokers (n=3, each).

2.6.4. Precision and Accuracy

Inter- and intra-assay precision and accuracy were calculated from quality control samples at three concentration levels (15, 25 and 37.5 µg/mL) by analyzing each QC in triplicate in three validation runs. Intra-assay precision and accuracy were evaluated for each quality control pool, by multiple analyses (n = 6) of the pool in one validation run.

2.6.5. Stability Experiments

Freeze/thaw stability was evaluated by analyzing of low QC (15 µg/mL) and high QC (37.5 µg/mL) over three freeze/thaw cycles (n = 3). Samples were frozen at -20 and thawed at room temperature. QC Samples for cycle one were frozen for at least 24 h then each consecutive cycle was frozen for at least 12 h before thawing at room temperature.

Analyte stability in thawed matrix was evaluated by removing two sets of low QC (15 µg/mL) and high QC (37.5 µg/mL) samples from -20°C storage, thawing and allowing to sit at room temperature for 6 and 24 h prior to extraction and analysis (n = 3).

Analyte stability in frozen matrix was evaluated by analyzing a sets of QC samples at low (15 µg/mL) and at high (37.5 µg/mL), QC samples. Samples were stored for 30 days at -20°C and analyzed versus freshly prepared calibration standards.

Post-preparative stability was evaluated at low (15 µg/mL) QC and high (37.5µg/mL) QC concentrations (n=3, each), Colored solutions were stored at room temperature for approximately 2 h then measured versus freshly prepared calibrators.

2.6.6. Dilution Integrity

The ability to dilute samples originally above the upper limit of the calibration range was validated by analyzing six replicate of 250 µg/mL QC sample as 10-fold dilutions with blank urine.

2.6.7. Inter-subject Variability

The effect of variable matrix effects from multiple individuals was investigated, quality control samples (n = 3) at a concentration of 15 µg/mL of tramadol hydrochloride were spiked into six different human blank urine sources, extracted and analyzed as described above.

3. Results and Discussion

3.1. Method Development

Different experimental variables were optimized to achieve maximum sensitivity. Initial experiments were carried out using diluted urine samples without any sample pretreatment; urine samples were diluted with buffer solution followed by ion pair formation. A significant interference was observed at 470 nm from endogenous urine components in blank samples. Sample pretreatment was found to be required in order to remove most of endogenous matrix components and avoid matrix interference.

3.1.1. Liquid-liquid Extraction

Liquid-liquid extraction methods are known to provide clean extracts in addition to simplicity and relatively low cost. Different organic solvents such as DCM, ethyl acetate, tert.-Butylmethyl ether were previously used to extract tramadol hydrochloride from biological matrices at high pH[4],[18],[32]. Most of liquid-liquid extraction methods include evaporation step of the organic solvent followed by reconstitution of dried extracts in the appropriate solvent. To avoid the evaporation step; different organic solvents such as DCM, ethyl acetate, benzene, toluene were tested in terms of analyte extraction from human urine and also solubility of the colored products. Ethyl acetate provide high extraction recovery and good solubility for the colored product, however, faint yellow color was observed in blank samples due to partial solubility of the reagent in ethyl acetate. DCM was found to be the solvent of choice for the proposed method; high extraction recovery and excellent solubility for colored product with colorless blank samples were obtained.

3.1.2. Effect of Sodium Hydroxide Concentration

Basic pH is essential for liquid-liquid extraction of tramadol hydrochloride from biological matrices to release the free base drug (non polar), increase solubility in organic solvent and maximize the extraction recovery. Effect of different NaOH concentrations (0.1 N, 1N and 2N) on extraction recovery was studied by comparing color intensities. 1.0 mL of 2N NaOH solution was found to be the optimum. Similar concentrations of hydrochloric acid (0.1 N, 1N and 2N) were used to neutralize sodium hydroxide before ion pair formation.

3.1.3. Effect of Reagent Concentration

The amount of tropeolin OOO required to develop maximum color intensity was found to be 1.5 mL of 0.1% (Figure. 3). Beyond this optimum volume of the reagent, the absorbance of the colored product decreased.

3.1.4. Effect of pH on Colored Product Intensity

The effect of pH on the color intensity was studied. It was found that acidic pH is required for the colored product

formation; no colored product was formed at basic pH and very low color intensity was observed in samples without buffer addition (neutral conditions). Different acidic buffers at pH (2.1, 3, 3.7 and 4) were compared and the pH yielding maximum absorbance was 3.7 (Figure. 4). Different volumes of acetate buffer pH 3.7 were also compared and 2.0 mL was found to be the optimum volume (Figure.5).

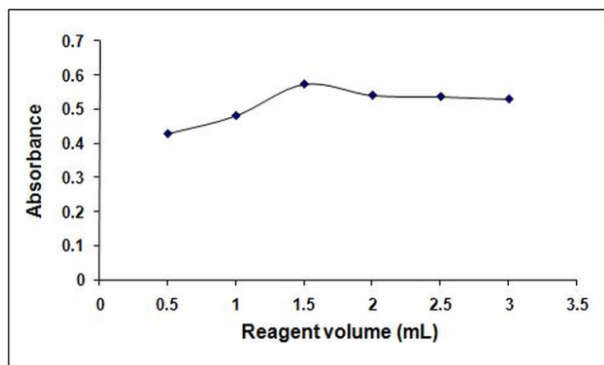


Figure 3. Effect of 0.1 % tropeolin OOO w/v w/v volume on the reaction of the dye with 30µg/mL tramadol

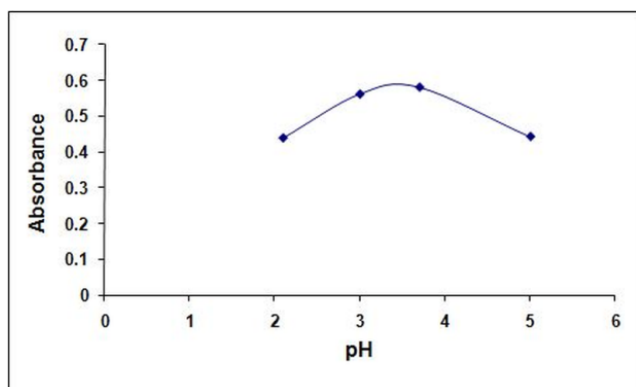


Figure 4. Effect of pH on the reaction of 30 µg/mL tramadol with 0.1 % tropeolin OOO w/v, w/v

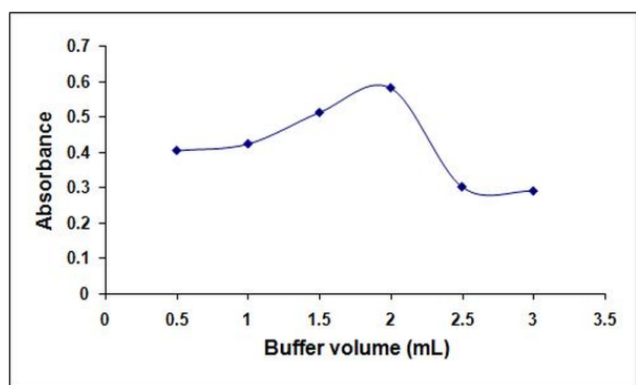


Figure 5. Effect of buffer volume (pH 3.7) on the reaction of 30 µg/mL tramadol with 0.1 % tropeolin OOO w/v, w/v

3.1.5. Reaction Chemistry and Stoichiometry

The anionic dye react with the drug cationic center at selected pH to produce a colored ion-pair chromagen that can be extracted with DCM (Figure 1). The reaction stoichiometry was determined using Job's method of

continuous variation[33]. The molar ratio of tramadol tropeolin OOO was found to be 1:1 (Figure. 6).

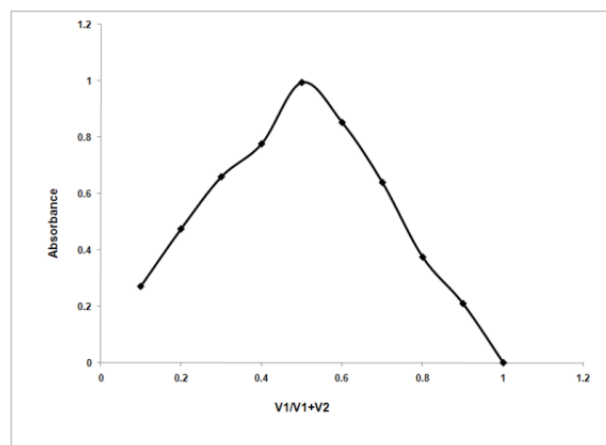


Figure 6. Determination of the reaction stoichiometry of tramadol (3×10^{-4} M) and Tropeolin OOO (3×10^{-4} M)

3.2. Validation

3.2.1. Linearity, LOQ and LOD

The instrument responses were plotted against tramadol concentrations to construct calibration curves. The calibration curve range was from 10 to 50 µg/mL and fit with a linear regression (figure 7). Two calibration curves were analyzed with each run on four separate days and the means of back-calculated standard values were used to evaluate the calibration data.

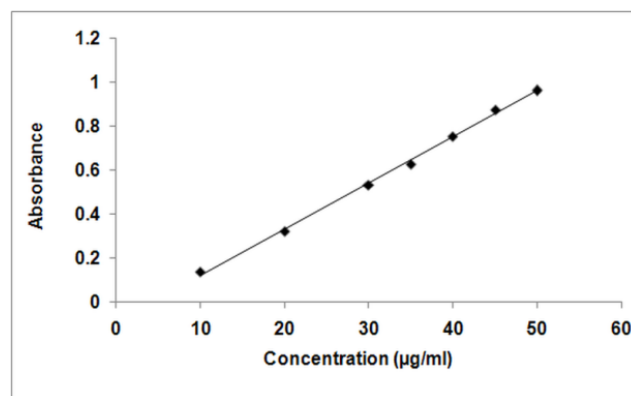


Figure 7. Calibration curve of tramadol hydrochloride

Table 1. Regression and Analytical Parameters

Parameters	Tramadol
λ max	470 nm
Linearity (µg/ml)	10-50
Limit of detection (µg/ml)	0.4
Limit of quantitaion (µg/ml)	1.2
Color stability (min)	120
Regression equation	
Slope \pm SD	0.023 ± 0.002
Intercept \pm SD	-0.122 ± 0.041
Correlation coefficient \pm SD	0.997 ± 0.001

The accuracy, represented as percentage difference from nominal values (%DFN), and precision, represented as percentage relative standard deviation (%RSD), ranged from 1.86 to 6.29 and from -0.09 to 7.32, respectively. The average curve parameters (slope, intercept and correlation coefficient) with standard deviation were 0.023 ± 0.002 , -0.122 ± 0.41 , and 0.997 ± 0.001 . The limit of detection (LOD) was 0.4 $\mu\text{g/mL}$ and the limit of quantitation was 1.2 $\mu\text{g/mL}$ (table. 1).

3.2.2. Selectivity

Six human urine samples from different individuals were extracted and evaluated for potential interferences with tramadol at 470 nm. No interference was observed. More experiments were performed during method development to evaluate potential interference from different analgesic, caffeine and nicotine. Acidic and weakly acidic compounds such as aspirin, ibuprofen, diclophenac sodium and piroxicam showed poor extraction recovery from urine samples under the proposed high pH liquid-liquid extraction procedure and no colored product was formed. Basic compounds such as acetaminophen and caffeine that can be extracted under the proposed conditions did not produce any colored product under the proposed conditions. Urine samples from a smoker individual (average 20 cigarettes per day) and three passive smokers were extracted to identify any potential interference from nicotine and its metabolites; No interference was observed. These experiments demonstrate the ability of the method to distinguish tramadol from the investigated interferences.

3.2.3. Precision and Accuracy

The inter-day precision (%RSD) and accuracy (%DFN) for low, medium and high quality control samples varied from 6.69 to 9.45 % and from -3.51 to 3.71%, respectively. The intra-day parameters for six replicates of low, medium, and high quality controls ranged from 3.89 to 6.22 %RSD (precision) and from -1.99 to 9.85 (accuracy). The precision and accuracy data for individual quality control samples are shown in Table. 2.

Table 2. Intra-day and Inter-day Precision and Accuracy of QC Samples

Parameters	Tramadol
λ_{max}	470 nm
Linearity ($\mu\text{g/mL}$)	10-50
Limit of detection ($\mu\text{g/mL}$)	0.4
Limit of quantitation ($\mu\text{g/mL}$)	1.2
Color stability (min)	120
Regression equation	
Slope \pm SD	0.023 ± 0.002
Intercept \pm SD	-0.122 ± 0.041
Correlation coefficient \pm SD	0.997 ± 0.001

The validity of the method to analyze urine samples with high concentrations of tramadol such in case of over dose, drug abuse, toxicity and postmortem sample analysis was evaluated. The intra-assay precision and accuracy of the

dilution quality control (250 $\mu\text{g/mL}$) samples were 8.88% and 9.65%, respectively.

3.2.4. Stability Experiments

Different stability experiments were performed with respect to precision and accuracy of the low and high quality control samples in triplicate analyses. A summary of all experiments is given

in Table 3. For freeze-thaw stability, samples were removed from -20°C storage and frozen and allowed to thaw on three separate days prior to analysis. The thawed matrix stability represents the two controls thawed on the bench top for 4 and 24 h before extraction. For the post preparative stability study, colored products were stored for 2 hours at room temperature. For all experiments the precision and accuracy were within $\pm 8.2\%$ (Table. 3).

Table 3. Precision and Accuracy Calculated from Stability Experiments

Experiment	RSD%	DFN%
Freeze/thaw stability (-20°C , 3 cycles)	<8.2	$<\pm 5.4$
Analyte stability in thawed matrix (4 h at RT)	<4.7	$<\pm 2.1$
Analyte stability in thawed matrix (24 h at RT)	<8.1	$<\pm 4.3$
Analyte stability in frozen matrix (30 days at -20°C)	<4.7	$<\pm 7.3$

3.2.5. Inter-subject Variability

Different endogenous matrix components from different individuals may affect the validity of the method. The effect of variable matrix effects from multiple individuals was investigated; all subjects showed acceptable accuracy and precision within $\pm 12\%$ (table. 4).

Table 4. Inter-Subject Precision and Accuracy of Low QC Samples (15 $\mu\text{g/mL}$)

Subject #	1	2	3	4	5	6
Mean	16.08	14.28	14.43	14.93	15.65	14.66
%RSD	9.01	0.68	12.06	5.12	5.48	8.35
%DFN	7.21	-4.78	-3.79	-0.44	4.31	-2.24

4. Conclusions

A simple and rapid method for determination of tramadol in human urine has been fully developed and validated using spectrophotometry. A single liquid-liquid extraction using DCM was employed and offered both extraction of target analyte from human urine then extraction of colored complex. The sensitivity (0.4 $\mu\text{g/mL}$ LOD), small sample size (3.0 mL), rapid, cheap and short analysis time make the method attractive for rapid clinical screening and quantitation of tramadol in human urine samples. LLE offers advantages of simplicity cleanness of the extracted sample and relatively low cost compared with other extraction methods such as solid-phase extraction. Tramadol was selectively extracted from human urine under conditions that removed most of endogenous urine

interference. No significant interference was observed from common analgesic, caffeine and smoking.

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