DEVELOPMENT AND VALIDATION OF A LC-MS /MS METHOD FOR THE QUANTITATIVE DETERMINATION OF TRAMADOL HYDROCHLORIDE

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DEVELOPMENT AND VALIDATION OF A LC-MS/MS METHOD FOR THE QUANTITATIVE DETERMINATION OF TRAMADOL HYDROCHLORIDE (Abstract): The **aim** of this study was to develop and validated a highly sensitive, accurate, and precise liquid chromatography and tandem mass spectrometry (LC/MS/MS) method for quantitative analysis of tramadol (TMH) in human plasma using metoprolol tartrate as internal standard (IS). **Material and methods:** Quantitative analysis was done with methanol and 0.15 % formic acid in water (40:60, v/v) as the mobile phase and C_{18} as the stationary phase with a flow rate of 0.5 mL/min and MS as the detector. The mass spectrometer was operated in positive polarity mode. The method was validated for specificity, sensitivity, precision, accuracy, and other analytical parameters. The level of tramadol data was statistically analyzed using ANOVA at a 95% confidence level. **Results:** The total analytical run time was 2.5 min. The results found were satisfactory over the linear calibration range of 10-500 ng/mL for TMH. It is a simple, fast, precise and accurate liquid chromatographic method. **Conclusions:** The developed method can be used by scientific community for the quantification of tramadol in plasma samples from various clinical studies *of different dose strengths.* **Keywords:** TRAMADOL, LC/MS/MS METHOD, QUANTIFICATION.

Pain is the most common symptom of injuries, illnesses, disorders and conditions that we can experience throughout our lives. Depending on the type of pain, different medications may be recommended to relieve the discomfort. One of the most used drugs worldwide for alleviating moderate to severe pain is tramadol (TMH). It presents a very good analgesic activity by its action on central nervous system. Tramadol is considered a weak opioid due to its relatively low affinity for μ-opioid receptor, the main target for traditional opioids (1). It is available in a variety of formulations such as oral, suppository and parenteral preparations. The drug tramadol is a racemic mixture of two enantiomers in the form of hydrochlorides. The racemic

mixture of (+) dextro and (-) levo enantiomer of tramadol has synergistic analgesic effect. Some studies show that tramadol is also an effective antidepressant (2). Tramadol, in combination with adrenaline, has been used as a local anaesthetic in maxillofacial surgery. After oral administration it is rapidly absorbed from the intestine. Its oral bioavailability is 65–70 % due to first-pass metabolism (3) and it is metabolized to O-desmethyltramadol (ODT) primarily by cytochrome P450 (CYP) 2D6, and N-desmethyltramadol (NDT) by CYP2B6 and CYP3A4. ODT and NDT are further metabolized to N,O-dides-methyltramadol (NODT) by CYPs (4). Tissue distribution of tramadol is about 2.6–2.9 L/kg body weight. Tramadol does not have serious side effects, it is not addictive in therapeutic doses, as seen in other opioids such as morphine (5). Tramadol has been determined by using several chromatographic techniques such as liquid chromatography (LC) with ultraviolet (6, 7) or fluorescence detection (8, 9), LC coupled to tandem mass spectrometry (MS/MS) (10-12), gas chromatography (13, 14). Drug analysis is important in the pharmaceutical industry because it can help in dosage form selection by studying the stability of the active compound and identifying impurities in pharmaceutical formulations. Analytical determinations of medicinal substances are not only applied in the process of manufacturing medicines, but also in forensics for the quantification of prohibited substances in doping or problems related to drug abuse. This study aimed to develop a rapid, simple determination method for tramadol in human plasma using an isocratic LC-MS/MS.

MATERIALS AND METHODS

Materials

Tramadol hydrochloride (TM) and metoprolol tartrate as an internal standard (IS) were obtained from Unichem Laboratories LTD (India). HPLC-grade methanol, formic acid, and trichloroacetic acid were purchased from Merck (Germany). All other reagents were of analytical grade and commercially available.

Solutions

Stock solutions of tramadol (10 μg/mL) and IS (100 ppm) were prepared in methanol. Standard solutions of tramadol were obtained by the dilution of stock solution with methanol.

Sample preparation

0.5 mL biologic sample was treated with 0.5 mL trichloroacetic acid 5% like deproteinized. The obtained solution was ultrasonicated for 5 minutes and then centrifugated at 4000 rpm for 10 minutes. The supernatants were filtrated with a Millex-LH syringe filter (0.45 μm, 4 mm, Merck Millipore Ltd., Billerica, MA, USA) before injection into the LC. From the supernatant, a volume of 5 μL was measured and analyzed under the conditions of the method.

Chromatographic conditions

Tramadol and metoprolol tartrate in the sample were separated using a Dionex Ultimate 3000 LC instrument. Separation was performed using a Hypurity™ Elite Hypersil C18 (100 x 3 mm, 3 μm). The mobile phase consisted of methanol and 0.15 % formic acid in water (40:60, v/v). The flow rate was 0.5 mL/min, and the column temperature was set at 40 °C, and the autoinjector was set at 4 °C.

Mass spectrometric conditions

The column effluent was monitored using a triple quadrupole mass spectrometer (3200 QTRAP®, AB Sciex, Foster City, CA, USA) equipped with an electrospray probe in positive ionization mode. The ion transitions were monitored using a dwell time of 200 milliseconds for each compound: tramadol, 264.4/58.3; metoprolol 268.1/121.18. Samples were introduced to the interface through a turbo ion spray with the temperature set at 300 °C. A high positive voltage of 3.8 kV was applied to the ion spray. The pressure of the ionization gas was 20 psi, and the pressure of the auxiliary gas was 30 psi. Collision energy for tramadol and metoprolol tartrate was 1.5 mTorr. The capillary temperature was set at 320°C.

Method validation

In order to determine the *specificity* of the method, the following chromatograms were recorded under the method conditions:

- blank solution - 0.5 mL of a biological sample from each source (S1-S6);

- sample solution - 0.5 mL of a biological sample (S1-S6) was mixed with 0.025 mL standard solution of 40 ppm concentration;

- internal standard solution - 0.5 mL biological sample (S1-S6) was mixed with 0.025 mL solution 40 ppm internal standard solution.

Selectivity was studied by investigating the magnitude of any interference caused by drug metabolites. In order to establish the influence of interferences, a number of drugs such as metoclopramide, famotidine, granisetron, pantoprazole and fentanyl were co-administered with tramadol. It was considered: Metoclopramide - ESI (-) 301.0 → 190.0 (15), Famotidine - ESI (+) 338.3 → 189.2 (16), Granisetron - ESI (+) 313.1 → 138.2 (17), Pantoprazole - ESI (+) 384.1 → 200.0 (18), Fentanyl - ESI (+) 337.3 → 188.26 (19).

The study of the residual effect on the column

Analyte traces that can be found in the samples are identified by observing similar signals. These signals must have an area that does not exceed 0.2 of the lowest concentration in the studied range, and in the case of components that are internal standards must not be greater than 5%.

Solutions used:

- blank solution - a volume of 0.5 mL of biological sample;

- sample solution at the upper limit of quantification (ULOQ) - 0.5 mL of biological sample was mixed with 0.025 mL of the standard solution of 40 ppm concentration;

- sample solution at the lower limit of quantification (LLOQ) - 0.5 mL biological sample was mixed with 0.02 mL 1000 ppb concentration standard solution.

- internal standard solution - 0.5 mL of a biological sample (S1-S6) was mixed with 0.05 mL of 40 ppm internal standard solution, resulting in a solution with a concentration of 1000 ppb.

The formula used to calculate the residual effect is shown below:

$$\%\, residual\ effect = \frac{signal\ area\ (analyte\ blank\ solution)}{signal\ area\ \ (analyte\ sample\ solution)\ (LLOQ)} \times 100$$

Linearity. For the study of the linearity of the method, 3 sets of working solutions were prepared, on the concentration range 10-500 ppb for tramadol (20). The internal standard used for each solution was 1000 ppb metoprolol tartrate (tab. I).

TABLE I.
Linearity of the method

	P1	P2	P3	P4	P5	P6
TMH (ppb)	10	30	100	250	375	500
MT (IS) (ppb)	1000	1000	1000	1000	1000	1000

The precision of the method

It was established by determining repeatability and intermediate precision. A number of 3 samples were prepared and analyzed for each concentration level (10, 100, and 500 ppb). The concentration for each individual sample was calculated based on the equation of the regression line and the values of the areas corresponding to the tramadol peaks (21). The obtained values were processed statistically and the value of the coefficient of variation (CV) was calculated.

Accuracy of the method

Three standard solutions of tramadol with different concentrations (table II) were used in order to evaluate the accuracy of the method (22, 23). For each concentration, 3 series of determinations were performed, and the degree of recovery was calculated with the relationship:

$$\%\ Recovery = \frac{Calculated\ Concentration\ Value}{Nominal\ Concentration} x100$$

TABLE II.
The solutions for the accuracy study

	3xLLOQ	50%	ULOQ
TMH (ppb)	30	250	350

Time stability of the analyzed samples concentrations

The stabilities of TMH and IS in plasma were assessed by comparing the areas measured over 7-60 hours of storage at room temperature with the initial peak area (20, 22).

RESULTS

In order to establish the optimal parameters of the method, preliminary tests were conducted. We selected the chromatographic column (Hypurity™ Elite Hypersil C18, 100 x 3 mm, 3 µm) after assessing three types of chromatography columns. The mobile phases recommended by the specialized literature consist of a mixture of methanol or acetonitrile with buffer solutions such as phosphate, triethylamine, acetate, and formic acid with a pH between 2.5 and 7.3.

The results obtained in the study of the specificity of the method are shown in table III.

The chromatograms corresponding to the compounds considered for the evaluation of interferences are presented in first figure.

TABLE III
The study of the specificity of the method

	Standard	Retention time	Area at LLOQ		Area at ULOQ
	TMH	1.75	283675		38572
	MT	1.71	9913		22079
Sample	**Retention time**	**Area at LOQ**	**Blank area**	**Calculated conc.**	**% Interference**
S1	1.75	0	0	0.00	0.00
	1.71	0	0	0.00	0.00
S2	1.75	0	0	0.00	0.00
	1.71	0	0	0.00	0.00
S3	1.75	0	0	0.00	0.00
	1.71	0	0	0.00	0.00
S4	1.75	0	0	0.00	0.00
	1.75	0	0	0.00	0.00
S5	1.71	0	0	0.00	0.00
	1.75	0	0	0.00	0.00
S6	1.71	0	0	0.00	0.00
	1.75	0	0	0.00	0.00

The results obtained in the study of the interferents are shown in table IV.

The obtained results for the study of the residual effect on the column, are reproduced in table V.

The data obtained at the stability of the minimum limit of quantification (LLOQ) are shown in table VI, where S/N is the ratio between the recorded signal (S) and the background noise (N).

TABLE IV.
The interference study caused by several concomitant analytes.

Compound	**Specific transition (m/z)**		**Areas ULOQ**	**Areas LLOQ**	**% Interference (compared to LLOQ)**
MT (IS)	268.1 → 121.18		44074		
Interferences	Specific transition (m/z)	Specific transition (det)	Areas (det)	Concentration (ng)	
	284.1→ 116.1	–	–	–	
Compound	Specific transition (m/z)		Areas ULOQ	Areas LLOQ	**% Interference (compared to LLOQ)**
TMH	268.1 → 121.18		44074		
Interferences	Specific transition (m/z)	Retention time (det)	Area (det)	Concentration (ng)	
Desmethyltramadol	250.1 → 58.1	–	–		

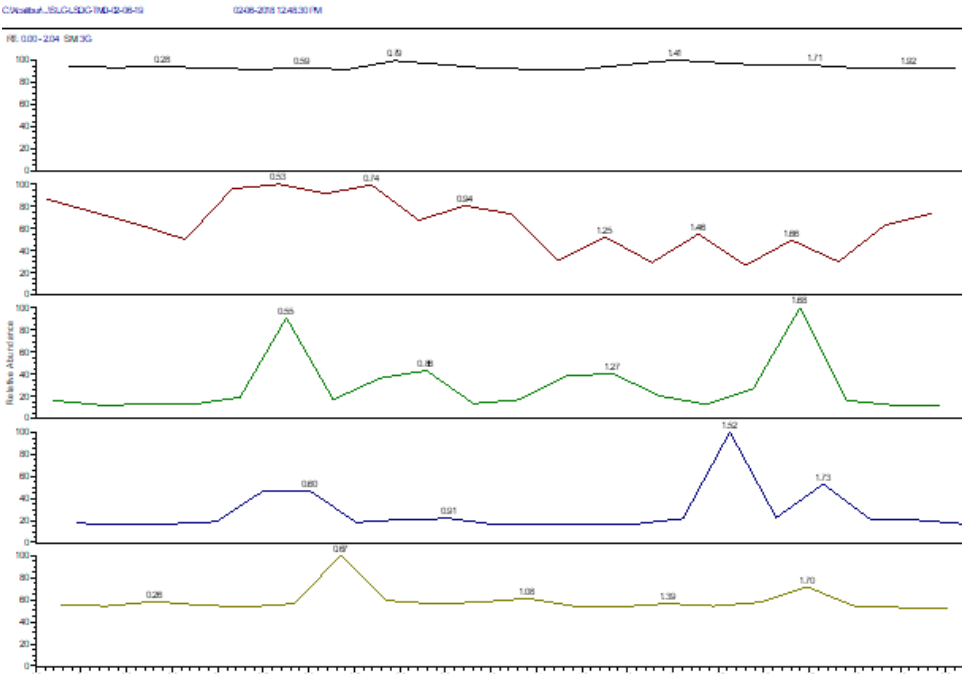

Fig 1. The chromatograms for metoclopramide, famotidine, granisetron, pantoprazole and fentanyl

TABLE V.
The residual effect on the column

Series	Sample	Areas	Residual effect (%) (against LLOQ)
TMH	Sample at ULOQ	**283675**	**0.0**
	Blank	**0**	
	Sample at LLOQ	**10337**	
MT	Blank	0	**0.0**
	Internal standard	44776	

TABLE VI.
Determination of the lower limit of quantification (LLOQ)

Name	Sample	S/N	Areas	Signal ratio LLOQ/BS
TMH	Sample at LLOQ	712	18151	0
	Blank sample	0	0	

The calibration curves obtained, through the graphical representation of the areas as a function of the tramadol concentration in the sample, are shown in figure 2.

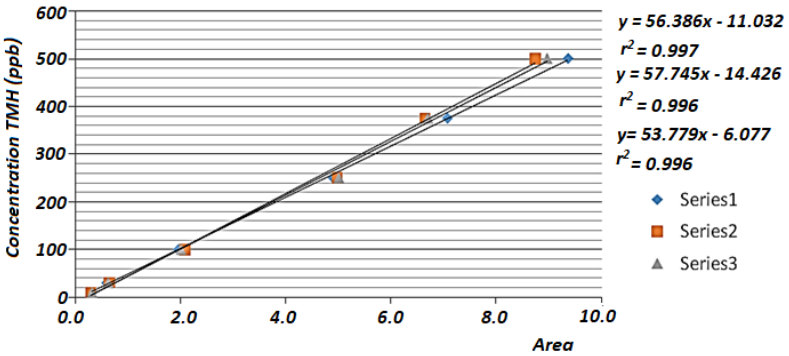

Fig. 2. Calibration curves for TMH

The experimental values obtained in the study of the precision of the method for the determination of tramadol hydrochloride by LC-MS/MS, are presented in table VII.

The graphical representation of the individual deviations for tramadol hydrochloride is shown in figure 3.

TABLE VII.

The precision of the method

TMH concentration (ppb)	10	100	500
Series	**S1**	**S2**	**S3**
day 1	93.45	96.27	103.78
day 1	98.92	93.28	90.90
day 2	107.63	91.74	99.17
day 2	109.74	99.40	103.19
Average	102.43	95.17	99.26
Standard deviation	7.60	3.39	5.94
CV	7.42	3.56	5.98

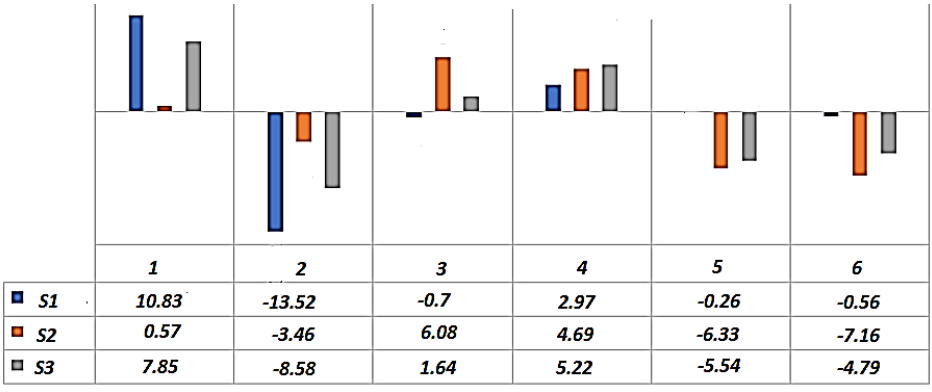

Fig. 3. Individual deviations of the calculated concentrations *vs.* theoretical concentrations of TMH

TABLE VIII.
Accuracy of the method

Theoretical Concentration (ppb)	Series	Area AS*	Area IS	Areas ratio AS/IS	Calculated Concentration (ng/mL)	Recovery (%)	RSD%
500	S1	235166	38973	6.03	327.07	93.45	1.25
100	S2	64454	33770	1.91	96.27	96.27	
10	S3	12068	32327	0.37	10.38	103.78	

***Mean of three replicates (n=3) *AS=analyzed solution *IS=internal standard**

The experimental values and calculations performed in order to evaluate the accuracy of the method for determining tramadol hydrochloride by LC/MS are presented in table VIII.

The results obtained in the tramadol stability study are presented in figure 4.

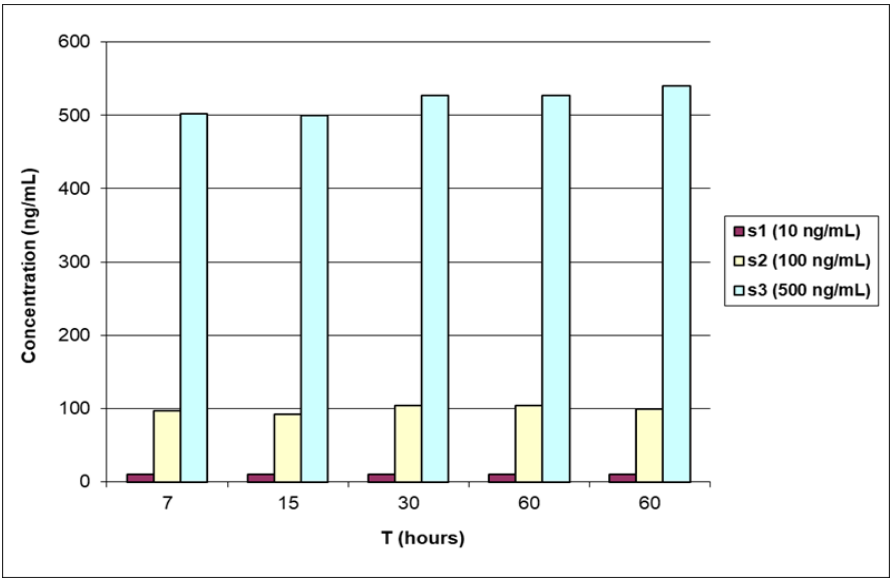

Fig. 4. Time stability of tramadol concentrations

DISCUSSION

The development of a practical chromatographic method for the determination of tramadol in human plasma is needed for clinical use. In our study, we used the MS/MS detection for tramadol because it possesses high sensitivity and selectivity. Ultraviolet detection from biological fluids is not suitable because of low sensitivity and selectivity (8). The LC separation of tramadol from a mixture using ultraviolet or fluorescence detection requires surfactants such as triethylamine and sodium dodecyl sulphate (9). These surfactants cause the ionic suppression of analytes in MS/MS analysis. The convenient ratio of the two solvents (formic acid : methanol) which provides good resolution was found

to be 40:60 (v/v). By changing this ratio, a decrease in peak area and height was found, and this would lead to a decrease in the sensitivity of the method. Other authors have used a mixture of acetonitrile - 0.1% formic acid 20 : 80 (v/v), as a mobile phase (8). The mobile phase consisted of methanol and a nonvolatile phosphoric acid salt solution was used by Ardakani *et al*. (24). The methods that are using a mobile phase with phosphate buffer are not suitable for MS/MS analysis.

In our experiments we followed the possibility of a simple procedure of processing samples, involving only deproteinization with 5% trichloroacetic acid. Other authors used for protein precipitation acetonitrile and methanol (25), or ethyl acetate (24). Using this procedure, the recoveries of tramadol in the present method were more than 89.9% while Ardakani *et al*. (23) obtained a recovery percentage of 80.8% and Tanaka *et al*. 85% (25). The run time for LC separation was shorter (5min) than in other studies (25, 26).

Analyzing table IV, it was observed the absence of signals that can be associated with interferents, and if they are present, then they are found in a concentration lower than 20% of the lower limit of quantification. Figure 2 showed that no interference occurred, due to the presence of the following substances: metoclopramide, granisetron, fentanyl, pantoprazole, and famotidine, administered simultaneously with tramadol.

In the column residual study, no residual signals were found for tramadol. For the control samples, no residual samples were found, after introducing the samples at the lower limit of quantification into the analysis (table V), as well as after using the samples at the upper limit of quantification.

The signals at the LLOQ for tramadol show a signal (S) to background noise (N) ratio of 712. On the other hand, the LLOQ value it must be a maximum of 5% of the ULOQ. In our case, it has 2% of the upper limit of quantification (table VI).

The calibration curve in human plasma of tramadol was linear over the concentration range of 10-500 ppb. Tanaka *et al*. reported a concentration range between 12.5-1600 ng/mL. The lower limit of quantification of tramadol was 10 ppb.

According to the data obtained and presented, the proposed analysis method is precise because the value of the coefficient of variation did not exceed 15% for the quality control samples, the maximum value being 7.42% (table VII). Other authors reported higher CV values for the precision study than in our case (12, 25).

The recovery of TMH was found to be in the range of 93.45-103.78%. These results show the proposed method is accurate. Complete data are shown in table VIII.

Analyzing the data in figure 4, the stability of the tramadol concentrations in the analyzed samples can be seen for 60 minutes.

CONCLUSIONS

We validated and applied a reverse phase LC method for the separation and quantitative determination, by mass spectrometry, of tramadol hydrochloride. The method was linear over the concentration range of 10-500 ppb tramadol hydrochloride. The value of the lower limit of quantification was at most 5% of the upper limit of quantification. The precision of the method estimated by repeatability and intermediate precision was below the 2% limit. The proposed method of analysis is accurate because the calculated concentration does not exceed 15% of the target values of the control samples. The shorter runtime allows the analysis of more sam-

ples. The method developed can be applied in drug analysis and control laboratories, toxicology laboratories, and for monitoring the concentration of tramadol in the blood. The developed method will be applied to evaluate the toxicity of tramadol on the human body as well as to evaluate the intensity of pain.

CONFLICT OF INTEREST AND FUNDING

The authors declare that there is no conflict of interest.

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