A COMPARISON OF FLUORESCENCE AND ULTRAVIOLET DETECTORS USING THE RP–HPLC TECHNIQUE FOR THE DETERMINATION OF TRAMADOL HYDROCHLORIDE (TRM-HCl): A CASE STUDY OF AL-BAYDA CITY, LIBYA

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Abstract

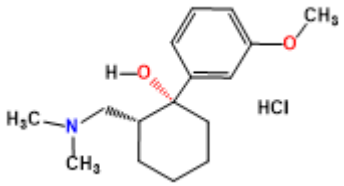
A fast and sensitive chromatographic RP–HPLC technique for examining tramadol hydrochloride (TRM-HCl) in tablets through using fluorescence detection (FL-D) and ultraviolet detection (UV-D) is reported in this paper. The separation was carried out using the reverse phase method on a Brownlee BIO C18 analytical column with a mobile phase consisting of 0.1% acetic acid and acetonitrile (2.5:7.5 v/v), which was pumped with an isocratic elution at a flow rate of 1 ml/min. The LOD and LQD values obtained in the current study indicate that FL-D is more sensitive, and hence preferable to UV detectors in the quantification of TRM-HCl tablets over the entire concentration range used (5-125 µg/ml). The study showed that the mean percentage recoveries from five samples were 99.93-100.023% (FL-D), which is somewhat similar to that of the UV-D (99.93-100.028). In conclusion, although Fl-D is acceptable for the quantification of tramadol tablets, UV-D offers higher detection sensitivity and reproducibility, particularly within concentrations that are low in the deposit collectors.

Keywords: Tramadol; RP-HPLC; UV-D; FL-D; Sensitivity.

Introduction

Tramadol hydrochloride (TRM-HCl), (1R,2R) -2- ((dimethylamino) methyl) -1- (3 methoxyphenyl) cyclohexan-1-ol (Scheme**1**), is a central analgesic with a low affinity for opioid receptors. It is used to alleviate moderate to severe pain (Dayer, Collart and Desmeules, 1994; Kanaan *et al.*, 2006; Abdel-Megied and Badr El-din, 2019; AlSamarrai, Abdoon and Hashim, 2019). TRM-HCl is a synthetic opioid agonist and serotonin reuptake inhibitor, whilst TRM is a safe drug for respiratory depression, cardiovascular and neurotransmission, unlike some other opioids (Klotz, 2003). This drug has two enantiomers and has a general

structure related to those of codeine and morphine. Interestingly, it has been reported that each enantiomer achieves analgesic activity through its own specific mechanism (Raffa et al., 1993).



Scheme (1): Structure of Tramadol Hydrochloride (TRM-HCl).

Unlike other morphine derivatives, the racemic combination of TRM-HCl has a powerful analgesic effect and tolerability compared to its individual enantiomers. This augmented analgesic activity is attributed to the enhanced inhibitory effects on pain transmission across the spinal cord through two different mechanisms (Budd and Langford, 1999), which comprise serotonin reuptake inhibition(Raffa et al.)via -TRM-HCl and/or epinephrine reuptake inhibition by (-)-TRM-HCl. TRM-HCl which is metabolized by the CP450 enzyme in the liver to form 11 metabolites. The (+)–TRM-HCl is the most predominant metabolite that acts as an μ-opioid agonist and has the advantage of low addiction, and is applied to relieve the pain caused in certain medical circumstances and by various diseases (e.g., for postoperative pain, dental pain, cancer, acute musculoskeletal pain, and as an adjuvant to patients with osteoarthritis and anxiety and depression) (Grond and Sablotzki, 2004; Salem et al., 2008; Organization, 2014, Clarot et al., 2003; Daubin et al., 2007). However, Although, TRM-HCl is mostly considered safe, a number of instances of administration of a lethal dose have been reported by the WHO, particularly in the Middle East, due to abuse. For this reason, the WHO has restricted several illegal tablet formulations (Goodarzi, Mehrpour and Eizadi-Mood, 2011; De Moraes et al., 2012; Dhillon, 2010; Raber and Momberger, 2002). Various analytical techniques have been employed to evaluate the quality of tramadol in pharmaceutical preparations, such as spectrophotometry (Küçük and Kadıoğlu, 2005; Rathore et al., 2009; Rajasekhar et al., 2011; Rajitha et al., 2011), the highly sensitive LC–MS/MS(Abdel-Megied and Badr El-din, 2019), spectrofluorometric techniques (Smith et al., 2008; Prabu et al., 2009), and HPLC coupled with UV (Kartinasari, Palupi and Indrayanto, 2004; Rajendraprasad et al., 2011; Kilaru et al., 2018) or FL (Overbeck and Blaschke, 1999; Saccomanni et al., 2010). As compared with the above techniques, high-resolution capillary GC has been less frequently used (Yilmaz and Erdem, 2015); indeed, there are few articles in the literature describing either the determination of tramadol in various matrices or of the high-performance thin-layer chromatography (HPTLC) determination of tramadol and its impurities in pharmaceutical

preparations (Krzek and Starek, 2004; Smith et al., 2008; Venkateshwarlu et al., 2008). In this context, Krzek and co-workers investigated performance separation on silica gel-coated chromatographic plates (HPTLC) using two mobile phases: (I) chloroform methanol-glacial acetic acid (9:2:0.1, v/v/v); and (II) chloroform-toluene-ethanol (9:8:1, v/v/v) (Krzek and Starek, 2004). However, these techniques can be time consuming, expensive, and often need some form of pre-treatment step. UV methods are very commonly used as detectors in HPLC analysis, but this approach is not very sensitive, especially when purine nucleosides are determined in biological samples. Nevertheless, UV detection is considered the most common method of detection in combination with HPLC for TRM determination (Patrolecco et al., 2013; Saravanan and Revathi, 2014; Giuliani et al., 2016). Hence, to improve the sensibility and reduce interference, an FL detector could be used to promote new techniques to assay TRM-HCl tablets as it will be described in this article. The aim of this study is to re-examine and validate similar HPLC procedures through using two different detectors (UV-D and FL-D) and by using the HPLC data of TRM-HCl tablets in Al-Bayda City, Libya as the case study. To investigate which detector is most sensitive and responsive to the presence of TRM-HCl, this work compares the use of separation with reverse phase RP-HPLC followed by FL detection to the one followed by UV detection. The results of the proposed techniques were established to be satisfactory and reliable, and the use of UV-D and FL-D did not show any considerable differences.

Experimental

Apparatus

Chromatographic separation was performed using a modular HPLC system: a Perkin Elmer HPLC Series PE-200 (USA), a Brownlee BIO C18 reversed-phase analytical column, 5 µm particle size, with dimensions 250 × 4.6 mm operating at ambient temperature, equipped with a P200 pump, solvent degasser DGU-3A, an automatic sampler AS200, a Rheodyne injector with a200 µl loop, with two types of detectors allowing the switch between either a UV-VIS detector (Series 200) for absorption measurements at 211 nm and/or a fluorescence detector (Series 200) for detecting light emission at $\lambda_{ex/em}$ = 272/298 nm with appropriate communication interface (Network Chromatography Interface NCI 900). All experiments were carried out in air at room temperature; final dataanalysis was performed using Microsoft Excel and Origin Lab Origin software.

Reagents and Solutions

All the chemicals used were supplied from Sigma Aldrich and CISME, Italy, without further purification. The solutions were prepared using double distilled water with a specific conductivity of 0.2 µS, as follows:

Tramadol HCl (TRM-HCl) stock solutions (1000 µg/ml) were prepared in high purity distilled water (100 ml) at room temperature. From this stock solution, working standard solutions

containing 5-25 µg/ml and 50- 125 µg/ml of TRM-HCl were prepared by serial dilutions for this study at room temperature.

Procedure for the Preparation of TRM-HCl Solution and Mobile Phase

Five TRM-HCl tablets (225 mg, Wave Pharmaceuticals Ltd) were powdered and mixed, and the powder (225 mg) then dissolved in 100 ml of double distilled water. The mixture solution was shaken well and filtered to remove any impurities in the remaining solution. The final volume was then adjusted with double distilled water; 10 µl aliquots of this solution were injected into the RP-HPLC by an auto injector. The average content of the tablets was determined by via comparison toa calibration curve. The mobile phase, consisting of acetic acid (CH_3COOH, 0.1%) and acetonitrile (MeCN) in a ratio of 2.5:7.5 v/v, was pumped in isocratic elution mode at a flow rate of 1 ml/min and with an injection volume of 10µl.

Results and Discussion

To establish the conditions for UV detection of TRM-HCl, the optimal UV wavelength of the detector had to be determined. The absorption spectra for TRM-HCl constituents presented in Figure (1a) closelyresemble each other. It is evident from this figure that the excitation wavelengths were 211, 265 and 272 nm Figure (1a). Since the excitation wavelengths of 211 and 265 nm are likely to be subject to significant interference, and there is no clear evidence for separate peaks at longer wavelength, thus the maximum absorbance at λ = 272 nm was chosen as the compromise optimum excitation wavelength Figure (1b). Based on this, appropriate experimental conditions were established for the FL-D used for the HPLC determination of TRM-HCl, and where the λ_{ex} and λ_{em} of 272 and 298 nm, respectively, of the fluorometers were the principal requirements for obtaining the maximum response (peak area counts) in the FL-D of TRM-HCl. The absorption and emission data recorded are consistent with those previously published in (Rathore et al., 2009; Smith et al., 2009; Zidane et al., 2019). Notably, any deviation from these chosen parameters yielded inconsistent results and lower sensitivities. In other words, instrumental setup could play an effect on sensitivity. UV-Vis spectrometers are was often horizontal in terms of incident beam, the sample, and the detector. FL spectrometers are was frequently at 90 degrees to observe the fluorescence from the sample without interference from the incident excitation beam. This suggests that the S/N would be better, yielding higher sensitivity. Various chromatographic conditions using different mobile phases (CH_3COOH/MeCN) were assessed to achieve good separation, resolution, and short run times. Optimal conditions were found as an isocratic elution using a column Brownlee BIO C18 type, 5µm particle size (250 × 4.6 mm). The various assays demonstrated that a proportion of CH_3COOH (0.1%)/MeCN (2.5:7.5v/v) was the most favourable, with TRM-HCl elution observed at ca. 4.15 min at a flow rate of 1.0 min/ml at aλ_{abs} of 211 nm and $\lambda_{ex/em}$ of 272/298 nm. Typical chromatograms are depicted in Figure (2).

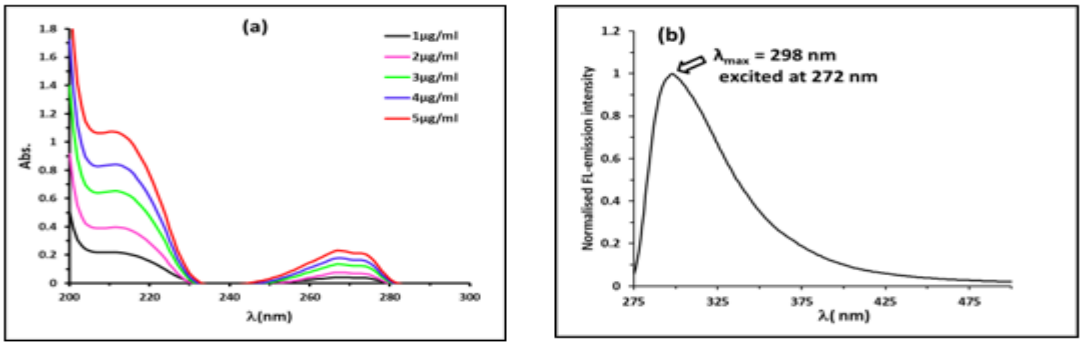


Figure (1): (a) Absorption Spectra for 1-5 µg/ml TRM-HCl, and (b) Normalized Fluorescence Spectrum for 5 µg/ml TRM-HCl in MeCN at Room Temperature in Air.

The injection volume was 10 µl for a room temperature working standard solution. Figure (2) illustrates the chromatographic response of TRM-HCl standards with selected concentrations of 25, 75 and 125 µg/ml using 10 µl injections obtained for FL-D and UV-D. The mean retention times (t_R, min) for the TRM-HCl in the two detectors were 4.11 mins. (FL-D), and 4.25 mins. (UV-D). The analyte peak obtained from each detector, depending on its sensitivity, was sharp and well defined; the most notable point was that as the concentration increased from 5 to 125 µg/ml, a gradual increase in the sensitivity was observed for both detectors. However, the baseline stability in the UV-D showed a broad band and, accordingly, the chromatographic efficiency was rather low compared to the two FL-D. The chromatograms of TRM-HCl extracts Figure (2) did not show any interfering peak corresponding to the t_R of TRM-HCl. The relatively high sensitivity of FL-D might be because the emission signal was measured over a low background level. This is inherently more sensitive than comparing two relatively large signals, as in the UV detector.

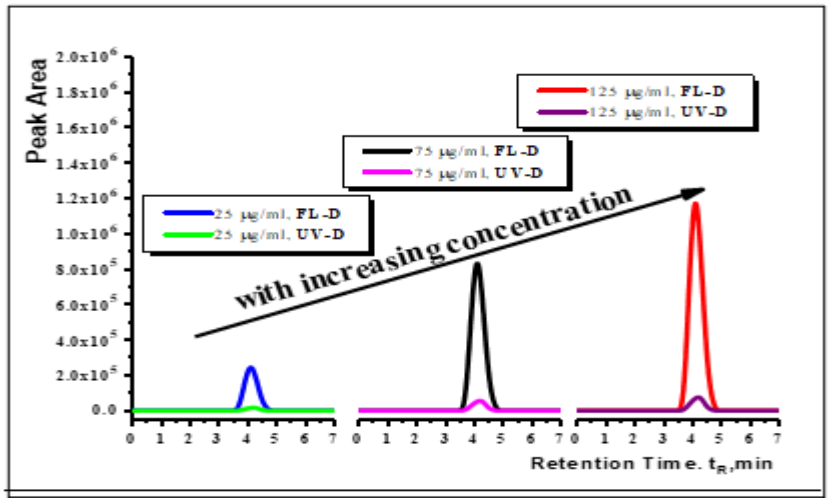


Figure (2): Representative Chromatograms Obtained from RP-HPLC FL-D and UV-D Studies (TRM-HCl, with Selected Concentrations; Injected: 10 µl; Flow Rate: 1.0 ml/min) at Room Temperature.

Calibration Curve and Linearity

A relationship between the average area count SD (n = 3) and the matching concentrations (5-25 and 50-125 µg/ml) for each of the detectors (UV-D and FL-D) was plotted for the investigated compound. The linear portion of each curve was fitted via simple linear regression, $y = ax + b$, where x is the standard solution concentration in µg/ml and the peak area count (y), gradient (a), and intercept (b), as shown in Figure (3), with the associated data reported in Table (1). For each of the detectors, the investigated compounds showed a linear relationship over the selected concentration range with $R^2 > 0.98$. High R^2 values for FL-D ($R^2 > 0.99$) were observed over the 50-125 µg/ml concentration range. These data are very similar to the literature data (0.997 for FL-D and 0.998 for UV-D).

As explained previously, the higher R^2 valuessuggest that this detector is relatively reliable in its response to a wide range of TRM-HCl concentrations compared to UV-D (Sundaram and Curry, 1997). The detector sensitivity indicated by the gradients of the straight lines illustrated in Figure 3 indicates that FL-D is very sensitive across all concentrations (range: 5-25 µg/ml or 50-125 µg/ml). The corresponding sensitivity ratio computed fromthe gradients is reported in Table (1).

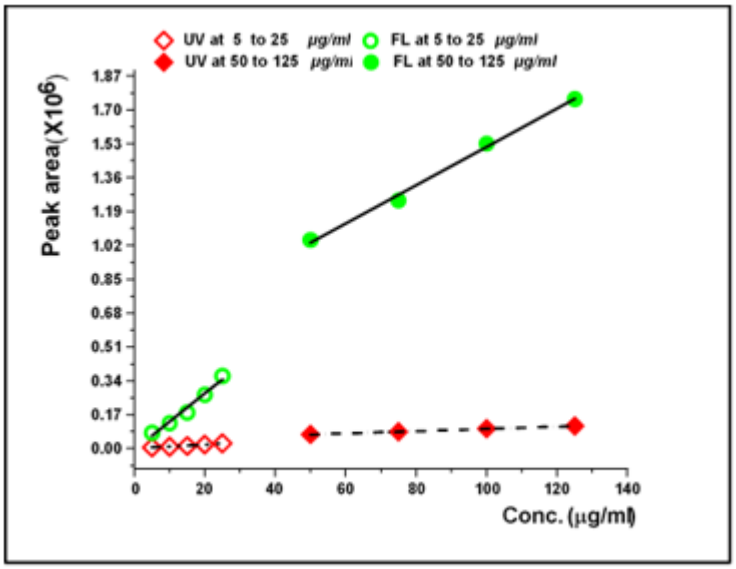


Figure (3): Calibration Curves for TRM-HCl Standards Using UV and FL Detectors After Post-Column Derivatization.

The limit of detection (LOD) and the limit of quantification (LOQ) for TRM-HCl were established for each of the detectors using the calibration standard. LODs and LOQs were calculated as analyte concentrations giving rise to signal-to-noise ratios of 3 and 10,

respectively, through the following expression, as suggested by the ICH standard (Swartz and Krull, 2018).

$$\mathbf{LOD} = \frac{S_{y/x}}{gradient} \times \mathbf{3.3} \quad \mathbf{(1)}$$

$$\mathbf{LOQ} = \frac{S_{y/x}}{gradient} \times \mathbf{10} \quad \mathbf{(2)}$$

Where $S_{y/x}$is defined as the standard deviation of the intercept (under the same conditions as for sample analysis), and the gradient of the calibration curve. The LODs and LOQs obtained for the FL-D and UV-D detectors for TRM-HCl over the two concentration ranges used (5-25 µg/ml and 50-125 µg/ml) are reported in Table (1).

Table (1): Regression Parameters for the Determination of TRM-HCl

Parameters	**FL-D**		**UV-D**	
Range, µg/ml	5-25	50-125	5-25	50-125
Gradient	14213.76	9637.80	1014.05	578.60
Intercept	-8544.68	550915	-218.74	41382.71
R^2	0.999	0.997	0.988	0.987
$S_{y/x}$	20852.60	25296.10	1415.62	936.59
LOD	4.84	8.66	4.61	5.34
LOQ	14.67	26.25	13.96	16.19
t_R, min	4.11		4.25	

Unexpectedly, when using FL-D, the LOD and LOQ values for TRM-HCl were higher than those found with UV-D. It is noteworthy that for both detectors the changes in the LODs and LOQs were determined at 5-25 µg/ml, which are surprisingly somewhat similar to those for the higher concentration range (50-125 µg/ml). The LODs for the FL-D and UV-D detectors changed over the 5-25 µg/ml concentration range, whilst further increases in concentration in the range 50-125 µg/ml led to a gradual increase in LODs by factors of *ca*. 1.78 and 1.15 for FL-D and UV-D, respectively see Table (1). In a similar manner, the LOQ for FL-D was determined to be 14.67, which is similar to that of the corresponding UV-D (13.96) in the 5-25 µg/ml concentration range, but in the higher concentration range (50-125 µg/ml) the LOQ for the FL-D detector was enhanced by *ca*. 1.62-fold compared tothe LOQ for the UV-D detector in the same concentration range. These data clearly suggest that FL-D increased the sensitivity of the method for the determination of the illegal use of TRM-HCl in water in a relative sense.

Precision and Accuracy

The precision and accuracy of the proposed technique was assessed by repeated analyses of the TRM-HCl solutions over the 5-125 μg/ml concentration range for each of the detectors.

The standard solutions were injected three times and the area counts for the three injections agreed within (Er %), showing good repeatability. The results which were expressed as a percentage relative standard deviation (RSD %) and a percentage relative error (Er %), and the associated data are reported in Table (2). For both detectors (FL-D and UV-D) the calculated RSD % and the Er % did not exceed 5.4%, demonstrating the high repeatability and accuracy of the experiment(Abdel-Megied and Badr El-din, 2019). It should be noted that at low concentrations the precision was high (low RSD %) for UV-D (average RSD % *ca*. 1.18 %), and low for FL-D (average RSD % *ca*. 2.44 %), whilst at high concentrations the precision was relatively high (low RSD%) for FL-D compared to the UV-D, see Table (2) entries 1-5 *vs*. 6-9. This is consistent with a work previously published by the Curry group using the liquid chromatographic method with different UV and FL detectors (Sundaram and Curry, 1997). The SD values were found to be between 861.3-16694.1 and 93.4-813.2 for FL-D and UV-D, respectively, over the concentrations range used, as shown in Table (2). This suggests that the degree of reproducibility, as given by the SD values, decreased in the order FL-D > UV-D. This observation is in agreement with previous reports in (Sundaram and Curry, 1997; Zhong and Que Hee, 2005; Díaz-Moroles *et al.*, 2007).

Table (2): Quality of Repeatability and Reproducibility Obtained Using the RP-HPLC Techniques with FL-D and UV-D

No.	µg/ ml	FL Detector					UV Detector				
		Area*	SD	RSD%	Found	RE %	Area*	SD	RSD%	Found	RE %
1	5	80249.0	4328.8	5.39	6.2470	4.9734	5930.2	116.5	1.96	6.0638	1.8767
2	10	128276.5	4217.3	3.28	9.6259	2.4226	9807.7	142.8	1.45	9.8876	0.9565
3	15	180241.1	2452.6	1.36	13.2819	0.9393	13167.2	93.4	0.71	13.2006	0.5018
4	20	269621.8	5320.0	1.97	19.5702	1.5280	19738.5	183.0	0.92	19.6808	0.7367
5	25	364920.4	861.3	0.23	26.2748	0.1979	26316.0	237.6	0.90	26.1672	0.7652
6	50	1046168.0	16694.1	1.59	51.3867	2.0378	70627.7	472.0	0.66	50.5447	1.3323
7	75	1245248.0	4786.4	0.38	72.0428	0.5406	83870.3	439.9	0.52	73.4320	0.8278
8	100	1531600.0	9246.0	0.60	101.7543	0.7833	100111.6	813.2	0.81	101.5020	1.1476
9	125	1753866.0	8596.4	0.49	124.8162	0.3766	113430.5	579.1	0.51	124.5213	0.3951
Average RE %						0.0751					0.0669

*The value is the mean of three determinations.

Application of the Technique to Real TRM-HCl Samplesin Illegal Tablet Forms

The mean percentage recovery for each fortification level obtained from each detector with its standard deviation (SD) and relative standard deviation (RSD), as recorded in Table (3), were derived from multiple injections of samples of each of the different tablet forms. This finding clearly showed that changing the detectors on the RP-HPLC had almost no effect on their recovery. The recovery studies for TRM-HCl tablet forms were found to be in the range of

99.93-100.023% and 99.93-100.028% for RP-HPLC using the FL-D and UV-D detectors, respectively, with the data for all five samples are reported in Table (3). It is worth noting that it is not required that 100% of the analyte is recovered, but the extent of the recovery should be consistent, precise, and reproducible. The mean percentage recoveries obtained from each detector in this study are in agreement with similar figures reported in the literature (96.33–100.92%) for the same tramadol hydrochloride pharmaceutical drugs (Donda *et al.*, 2016; Abdel-Megied and Badr El-din, 2019). Moreover, the method proved to be precise for RSD % *ca*. ≤ 1%, whilst the highest RSD % was *ca*. 0.00118% for FL-D and 0.00102% for UV-D which indicates that the method is applicable to non-dosage forms as these results are consistent with other research, in the literature, dealing with tramadol studies carried out on commercial and pharmaceutical forms (Smith *et al.*, 2008; Saccomanni *et al.*, 2010; Setty, 2012; AlSamarrai, Abdoon and Hashim, 2019).

Table (3): Application of the Suggested Technique for Assay of TRM-HCl in Illegal Tablet Forms

No.	Tablet Forms	TTA, Mg	FL-D				UV-D			
			Found* mg	SD	RSD (%)	Mean Recovery (%)	Found* Mg	SD	RSD (%)	Mean Recovery (%)
1	Tarmol-X	225	224.844	0.00266	0.00118	99.93	224.849	0.00193	0.00086	99.93
2	Super Tramadol -X	225	224.881	0.00072	0.00032	99.94	224.849	0.00086	0.00038	99.95
3	Super Tramadol -Quick Action	225	224.956	0.00176	0.00078	99.98	224.959	0.00229	0.00102	99.98
4	Super Tramadol Tablets	225	224.969	0.00081	0.00036	99.98	224.972	0.00103	0.000458	99.99
5	Tamol-X	225	225.051	0.00003	0.00001	100.023	225.064	0.00065	0.000288	100.028

TTA = Theoretical Taking amount, mg

***Average of Triplicate Measurements, SD = Standard Deviation, RSD = Relative Standard Deviation**

Conclusion

The following conclusions can be drawn from the above: to the best of our knowledge, the present study is the first report of FL and UV detector characteristics studying TRM-HCl tablets used as illegal drugs. Both FL-D and UV-D allowed all five TRM-HCl concentrations to be detected. Though the FL-D technique did not detect interference from the hydrolysate blank, UV-D technique did. TRM-HCl could be detected at higher sensitivities by FL-D than

by UV, and consequently produced clear chromatograms at different concentrations. Moreover, FL detection enhanced the specificity of the method, and the sensitivity as well within the range necessary for the detection of levels normally present in the deficiency states encountered in other species. Furthermore, the method for the determination of TRM-HCl deposit collectors reported in this paper was simple and relatively shorter and simpler than a similar method reported in the previous literature (Thompson *et al.*, 1989; Nobilis *et al.*, 2003), because pre-concentration by anion-exchange chromatography was unnecessary. Using the FL detector, the LOD and LOQ values for TRM-HCl were relatively higher than those found via UV detection, which clearly demonstrated that FL detection relatively increased the sensitivity of the technique. Although the sensitivity of fluorescence techniques can be as much as 1000 times greater than that of absorption spectroscopy, our study showed that the use of UV-D would be acceptable for the quantification of TRM-HCl. The method was found to be simple, precise, accurate, specific, and with a linear response over the concentration range tested (5-25 and 50-125 µg/ml) with a correlation coefficient >0.987 for the two detectors (FL-D and UV-D. The use of analytical conditions and the mobile phase system for the determination of TRM-HCl samples offer a particular advantage in that the rate of analysis can be increased without any apparent reduction in sensitivity or reproducibility within a short analysis time. These chromatographic conditions may also be compatible with other detection systems. This suggests that the method has many attractive merits, such as simplicity, sufficient sensitivity, high specificity, and rapid measurement times for TRM-HCl formulations in non-pharmaceutical preparations. Lastly, this technique does not require complex procedures such as sample extraction and/or sample cleaning, and does not require large volumes of samples and solvents. The results revealed that the applied method appears promising for the differentiation of genuine tramadol tablets from counterfeit ones without the need for prior separation. Therefore, this process can be proposed for routine analyses in laboratories and for the purposes of quality control.

Acknowledgements

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Conflicts of interest

The researchers declare that there is no conflict of interest regarding the publication of this article.

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