

## Article

# Development and Validation of Liquid Chromatography-Tandem Mass Spectrometry Method for Simultaneous Determination of Tramadol and Its Phase I and II Metabolites in Human Urine

Varsha Chauhan <sup>1</sup>, Manu Sharma <sup>1,\*</sup>, Abhishek Tiwari <sup>2</sup>, Varsha Tiwari <sup>2</sup>, Manish Kumar <sup>3,\*</sup>, Tarun Virmani <sup>4</sup>, Girish Kumar <sup>4</sup>, Najla Altwaijry <sup>5</sup>, Omkulthom Al kamaly <sup>5</sup>, Asmaa Saleh <sup>5</sup> and Abdulsalam Alhalmi <sup>6</sup>

<sup>1</sup> Department of Chemistry, National Forensic Sciences University, Ministry of Home Affairs, Sec-3, Rohini, New Delhi 110085, India; varshachauhan821@gmail.com

<sup>2</sup> Pharmacy Academy, IFTM University, Lodhipur-Rajpur, Moradabad 244102, India; abhishekt1983@gmail.com (A.T.); varshat1983@gmail.com (V.T.)

<sup>3</sup> School of Pharmaceutical Sciences, CT University, Ludhiana 142024, India

<sup>4</sup> School of Pharmaceutical Sciences, MVN University, Palwal 121105, India; tarun.virmani@mvn.edu.in (T.V.); girish.kumar@mvn.edu.in (G.K.)

<sup>5</sup> Department of Pharmaceutical Sciences, College of Pharmacy, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia; naaltwaijry@pnu.edu.sa (N.A.); omalkmali@pnu.edu.sa (O.A.); asali@pnu.edu.sa (A.S.)

<sup>6</sup> Department of Pharmaceutics School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi 110062, India; asalamahmed5@gmail.com

\* Correspondence: lantadene@hotmail.com (M.S.); manish\_singh17@rediffmail.com (M.K.)



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**Abstract:** Tramadol (TD) has been prescribed frequently in many countries for more than 40 years, but there is a risk of its misuse and trafficking. As a result, drug analysis has numerous legal and socially relevant implications, making it an essential part of modern analytical chemistry. Thus, the method for the detection of TD and its phase I and phase II metabolites in human urine has been developed and validated using a rapid and efficient approach combining liquid chromatography-tandem mass spectrometry (LC-MS/MS) with *electrospray ionization*. The sample preparation was best performed using dispersive liquid-liquid microextraction. Analysis was performed using an HyPRITY C18 column, and isocratic elution with methanol: water (35:65) with 0.2% formic acid was used. TD and its metabolites were detected at 264.2 (TD/M0) with a base peak at 58.2, 250.3758 (M1), 250.3124 (M2), 236.3976 (M3), 222.5361 (M4), and 236.4475 (M5) *m/z* peaks. TD showed linearity between 0.1 and 160 ng/mL ( $R^2 = 0.9981$ ). The accuracy ranged from 95.56 to 100.21% for the three concentration levels, while the between- and within-day RSD ranged from 1.58 to 3.92%. The absolute TD recovery was 96.29, 96.91, and 94.31% for the concentrations of 5, 50, and 150 ng/mL, respectively. TD's phase I metabolites, M1–5 along with nine phase II metabolites, such as sulfo- and glucurono-conjugated metabolites, oxidative TD derivatives, and sulfo-conjugated metabolites were also identified in the urine samples. The pharmacokinetics and metabolism data given provide information for the design of possible future research disorders, evaluating drug mechanism and neurotoxicity and for the effective application screening of TD.

**Keywords:** Tramadol; metabolites; achiral; tandem mass spectrometry; toxicology; urine matrix

## 1. Introduction

TD is a 2-(dimethylaminomethyl)-1-(3-methoxyphenyl) cyclohexanol or 4-phenylpiperidine analogue of codeine. TD, a widely prescribed analgesic, has gained significant attention in the field of pain management due to its efficacy in alleviating moderate to severe pain [1]. TD may provide a euphoric effect comparable to oxycodone when taken orally in large dosages. The predominant causes of Tramadol poisoning have been identified

as suicide attempts (52–80%), followed by abuse (18–31%), and accidental intoxication (1–11%). At least 20% of instances of TD poisoning were associated with a history of chronic TD misuse or opioid dependency [2]. As a prodrug, TD undergoes extensive metabolism in the liver. Understanding the metabolic profile of TD is crucial for evaluating its pharmacokinetics, therapeutic monitoring, drug interactions, and forensic analysis [3]. It is frequently taken orally and is quickly absorbed completely, with distribution in two hours. TD has a therapeutic blood concentration range of 0.1 to 0.3 mg/L. Moreover, blood values of 1 and 2 mg/L have been described as potentially dangerous and fatal, respectively [4,5].

TD has around 20% plasma protein-binding and a therapeutic plasma concentration. When taken orally, TD is rapidly absorbed and has a 65–70% bioavailability. The elimination of TD from the human body primarily occurs via renal excretion after hepatic metabolism through N- and O-demethylation processes, facilitated via cytochrome P450 pathways, particularly CYP2D6. As a result, overdose or intoxication might result from a deficiency in these systems or the concurrent use of other drugs processed via the same hepatic pathways. TD’s analgesic effectiveness is primarily due to ODT (O-desmethyl TD), a pharmacologically active metabolite. Around 10–30% of the parent dosage in the urine is excreted unaltered [6,7]. TD and M1 (ODT) have half-lives of 5–6 and 7–9 h, respectively. Thus, regular dosage may cause bioaccumulation due to M1’s extended elimination half-life [8]. The six metabolic pathways that inculcate N-demethylation, O-demethylation, oxidative N-dealkylation, dehydration, cyclohexyl oxidation, and conjugation yield TD metabolites [9]. The primary metabolites are M5 (N,O-desmethyl TD) and its conjugates, M1 and its conjugates, and M2 (N-desmethyl TD), whereas M3 (N,N-desmethyl TD) and M4 (N,N,O-desmethyl TD) and their conjugates are only formed in minimum concentrations. Phase I reactions yield 11 metabolites, whereas phase II reactions generate 12, and the most active metabolite is M1 (Figure 1) [10].

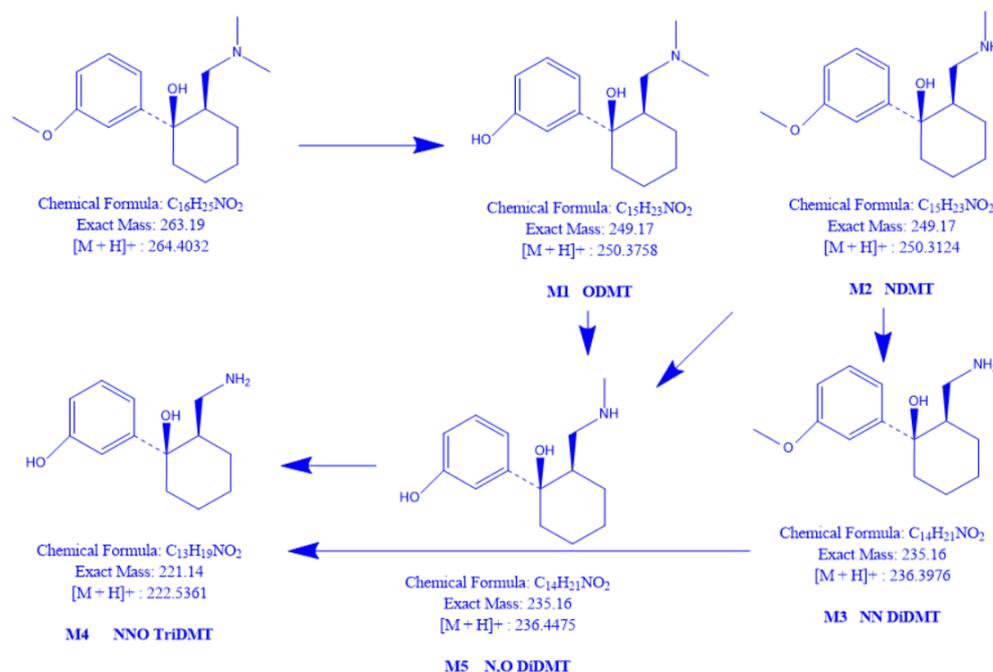


Figure 1. Illustrates the metabolic pathway of TD.

Drugs only persist in various matrices for a brief period of time. Drugs can be detected in both saliva and blood for a period of time of 24 h. Drugs can be traced in urine for up to 40 h and in hair samples for up to 90 days. When comparing urine to other biological samples, such as blood or plasma, TD and its metabolites generally occur in higher concentrations in urine. The identification and accurate quantification of these substances may be made possible by the increased concentration. Additionally, compared

to other matrices that might have enzymatic activity or undergo fast metabolism, urine samples are very stable. Compared to other sample collection techniques, including blood or tissue sampling, collecting urine is a non-invasive and very simple procedure. Large quantities of it are easily obtainable, facilitating repeated or longitudinal testing [11].

TD is a controlled substance in many jurisdictions, and its abuse potential has led to its inclusion in drug testing panels. Testing for TD metabolites can help identify TD use and differentiate it from other opioids or drugs of abuse, aiding in forensic investigations and workplace drug testing programs. Thus, the analysis of TD metabolites is important for understanding the drug's pharmacokinetics, monitoring therapeutic response, assessing drug interactions, investigating overdoses, and conducting forensic analyses.

For TD and its metabolites analysis, numerous HPLC methodologies using fluorescence [12], ultraviolet [13], MS detection [14], and electrochemical [15,16] have been developed. Chiral high-performance liquid chromatography (HPLC) detection methods help to analyze TD and its metabolite enantiomeric ratios [17,18]. As per the previously reported studies, the chiral column was used to identify and separate TD, M1, and M2 metabolites in the plasma and urine samples [19–22]. TD misuse or overdose can lead to severe adverse effects, including respiratory depression, seizures, and even death. The identification of phase I and phase II metabolites is vital in toxicology investigations to determine the extent of TD exposure and assess the contribution of specific metabolites to toxicity. By identifying and quantifying these metabolites in biological samples, such as urine or blood, toxicologists can gain insights into the metabolic pathways involved in TD toxicity and facilitate appropriate medical interventions. Chiral columns have their advantages for the enantiomeric separation of TD, they also present disadvantages such as limited applicability, increased method complexity, higher cost, reduced column lifetime, and lack of universality. However, achiral analytical techniques have also emerged in pharmacokinetic and bioequivalence assessments and forensic investigations. In contrast, achiral columns offer advantages such as broader applicability, simpler method development, and cost-effectiveness. They can be used for the separation of a wide range of compounds, including TD and its metabolites, without the need for enantiomeric resolution. Achiral columns are generally more robust, exhibit longer column lifetimes, and provide greater flexibility in method development and optimization. There is no such significant achiral approach in human urine for the identification and separation of TD and its metabolites (M1, M2, M3, M4, and M5) [23–25].

In the present study, we aimed to develop and validate a robust LC-MS/MS method for the simultaneous determination of TD and its phase I and II metabolites in human urine. The developed method offers several advantages over conventional analytical techniques, including enhanced sensitivity, specificity, and efficiency in detecting and quantifying multiple analytes within a single analytical run. In particular, the determination of TD and its phase I and II metabolites in human urine is of great importance due to their clinical significance and potential abuse. However, the analytical determinations of these compounds have broad applications, extending beyond drug manufacturing to include forensic science and the quantification of banned substances in cases related to doping or drug abuse. The separation of TD and metabolites via chromatography was acceptable, resulting in a quick turnaround for the analysis. The study successfully investigated the parent and metabolites of a 50 mg TD dosage in urine.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Acetonitrile, methanol, formic acid, chloroform, diethyl ether, and ethyl acetate of HPLC grade were procured from Merck, India. The reference standards of TD hydrochloride were procured from the Indian Pharmacopoeia Commission, India. The formulations of (Tramzac) TD hydrochloride was procured from Janssen Pharmaceuticals and Zydus Cadilla, India.

## 2.2. Urine Sampling

Four volunteers were considered since these individuals were already on TD. The volunteers were from the age group of 25–30 and they were prescribed TD by their doctors. The individuals were briefed about the study's objective and consented to participate. The volunteers were prohibited from taking any other drug for the preceding 24 h. The participants fasted for 12 h before ingesting 50 mg TD (a 50 mg Tramazac capsule) with water and then fasted for another 2 h. After collecting the last urine samples, the individuals were monitored for up to 24 h.

## 2.3. Sample Collection

Before the intake of TD at 2, 4, 6, 8, 10, and 12 h after the dose, urine samples were collected. For analysis, 10 mL of the urine was collected, centrifuged, decanted, and kept at  $-4\text{ }^{\circ}\text{C}$ .

## 2.4. Preparation of Standard and Quality Control Samples

Accurately weighed standard was dissolved in  $\text{CH}_3\text{OH}$ , and a stock solution of 1 mg/mL TD standard was prepared. The working solution was made by diluting the solution with  $\text{CH}_3\text{OH}$  and then putting it in the fridge at  $-4\text{ }^{\circ}\text{C}$ . TD concentrations in the range of 0.1 to 160.00 ng/mL were used to create the calibration curves. The quality control (QC) samples were made from blank urine and spiked with three concentrations of individually prepared stock standard solutions.

## 2.5. Sample Preparation and Extraction Procedure

Various combinations of liquid–liquid extraction (LLE) and dispersive liquid–liquid microextraction (DLLME) were used to improve the extraction's effectiveness. The DLLME approach was found to be superior to LLE in this study. Various solvents (ethyl acetate, diethyl ether,  $\text{CH}_2\text{Cl}_2$ ,  $\text{CH}_3\text{CN}$ ,  $\text{C}_4\text{H}_9\text{OH}$ , and  $\text{CHCl}_3$ ) were examined for DLLME. Lastly, 0.5 mL of pre-treated urine was injected with a dispersive and binary extraction solvent combination (ethyl acetate and chloroform (1:2)). A turbid solution was created immediately. The dispersed tiny droplets of chloroform and ethyl acetate were collected after the cloudy solution was centrifuged for ten minutes at 4500 rpm. After being dried, the phase that had been deposited was moved to another glass tube.

## 2.6. Analytical Conditions

### 2.6.1. Instrumental Conditions

A Thermo Scientific, India, Accela HPLC System with an Accela Pump 1250 and an Accela Autosampler Plus SRVYR-ASP interfaced to a Thermo Scientific TSQ Quantum Access MAX with an ESI interface was used for the LC-MS/MS analysis. LCQUAN<sup>TM</sup> quantitation software 2.6.0.1128 and TSQ<sup>TM</sup> tune master software 2.3.0.1206 version SP1, India were used to acquire chromatograms and mass spectra, respectively.

### 2.6.2. Chromatographic Conditions

Mobile phase equipment and an analytical Thermo Fisher Scientific HyPURITY RP C18 ( $150 \times 4.6\text{ mm ID}$ ,  $5\text{ }\mu\text{m}$ ) column were utilized for chromatographic separation. The mobile phase of  $\text{CH}_3\text{OH}:\text{H}_2\text{O}$  (35:65 *v/v*) with 0.2%  $\text{HCOOH}$  has been used. The analyte was eluted at a flow rate of  $500\text{ }\mu\text{L}/\text{min}$ . The run was scheduled to last ten minutes. For optimal sensitivity, the auxiliary gas pressure of 20 V, the spray voltage of 4525 V, the temperature of the vaporizer of  $350\text{ }^{\circ}\text{C}$ , the sheath gas pressure of 55 psi, and the capillary temperature of  $350\text{ }^{\circ}\text{C}$  was chosen. Data were collected for mass spectrometry between 2.5 and 10.0 min. Post-column splitting was used to divert column eluent for disposal of the remaining time.

### 2.6.3. Pharmacokinetic Analysis of TD in Human Urine

Before the medicine was administered (pre-injection: time 0) at 2, 4, 6, 8, 10, 12, and 24 h after a single 50 mg dosage, urine samples were collected and stored at  $-4\text{ }^{\circ}\text{C}$ . Urine samples were extracted following the aforementioned method. Each sample was introduced into the LC/MS/MS apparatus in an aliquot volume of  $5\text{ }\mu\text{L}$ .

### 2.7. Method Validation

A total of five urine samples, both with and without an internal standard to account for interference peaks. The amount of TD or IS (TD-d6  $50\text{ ng/mL}$ ) that could be extracted from urine was estimated by adding TD or IS in triplicate to a sample of blank urine. These sample's total peak areas were compared to a reference sample at each concentration. The reference sample was extracted from blank urine, mixed with the drugs, and processed as previously mentioned in triplicate for each concentration. After injecting multiple TD standard solutions into urine blanks and processing the samples, calibration curves for eleven urine concentration levels were produced. TD concentrations ( $x$ ) spiked in blank urine were compared to the drug's peak area ratio ( $y$ ).

The spectrum of QC sample concentrations was examined in sextuplets to establish intra-day accuracy and precision. The sextuplets' peak area ratios were used to measure precision. Each sample's concentration and percentage deviation was determined from the previous calibration curve. The precision and accuracy of QC samples were determined thrice. The percentage change between each QC's nominal and mean concentrations was used to estimate the accuracy for up to three days. The detection limit was set as the lowest concentration of TD that could be found with a signal-to-noise ratio of three-to-one. The limit of quantitation was determined based on the calibration curve's lowest concentration. Three LOQ samples were examined in triplicate for three days. Three distinct concentrations of standard quality control samples were frozen at  $4\text{ }^{\circ}\text{C}$  from a fresh stock solution of drug-free urine analytes.

## 3. Results and Discussion

### 3.1. Discussion on TD

TD and its metabolites can be simultaneously identified and separated, allowing for a comprehensive analysis of the parent drug and all of its different metabolites in a single analytical run. Various phase I and II metabolites are produced as a result of TD's broad metabolism in the body. TD's pharmacokinetics, metabolism, and forensic/toxicological applications have all benefited from the development of an approach for the simultaneous identification and separation of TD and its phase I and II metabolites from the biological matrices. Thus, the methodology was developed to identify and separate the maximum number of TD metabolites from the single dosage of TD from urine samples because the majority of the previously developed methodologies were focused on the identification of TD, ODT, and NDT.

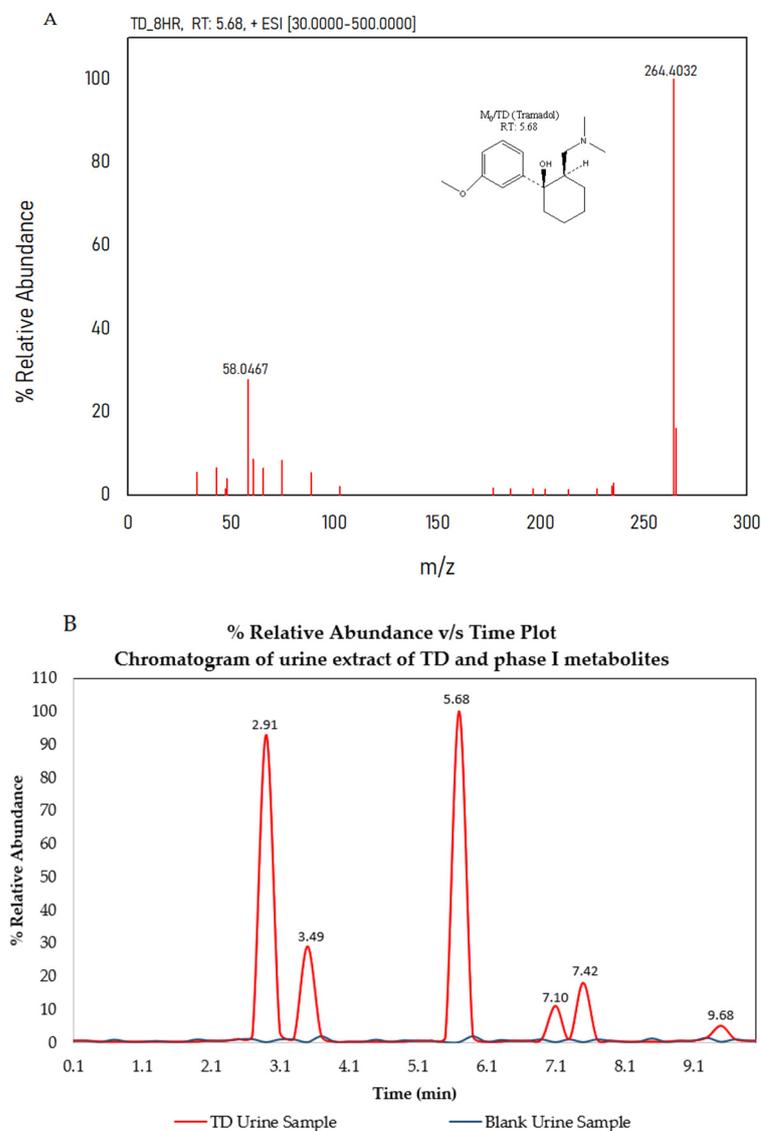
### 3.2. Method Validation

#### 3.2.1. Optimization of LC Conditions

One of the essential factors in the chromatographic analysis is the mobile phase. The mobile phase's constituents can significantly affect the method's sensitivity, retention time, and analyte separation. The current study intended to establish a method for the simultaneous detection of TD and its metabolites, employing DLLME extraction as a sample preparation methodology, with the targeted molecule separated using an achiral C18 column. MS-MS detection was then utilized. HyPURITY C18 ( $150 \times 4.6\text{ mm ID } 5\text{ }\mu\text{m}$ ) analytical achiral column was employed with a mobile phase apparatus to separate chromatographically.

Formic acid at various concentrations and different ratios of organic solvents (methanol/ acetonitrile) were studied. Columns were also examined for the best sensitivity, resolution, and retention time. Several mobile phases were tried in order to separate all the targeted

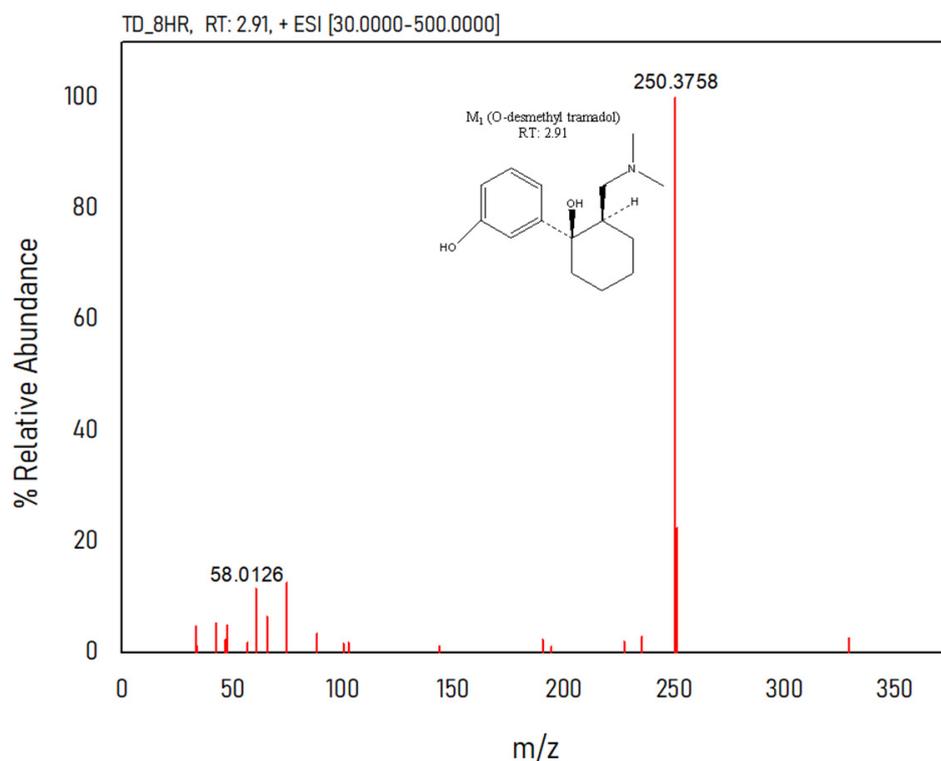
analytes. The optimum separation conditions for TD and its phase I and II metabolites involve utilizing 0.2% formic acid along with a mixture of CH<sub>3</sub>OH and H<sub>2</sub>O in a ratio of 35:65 (Figure 2). An operation rate of 500  $\mu$ L/min for analyte elution was utilized, and ten minutes were allocated for the runtime.



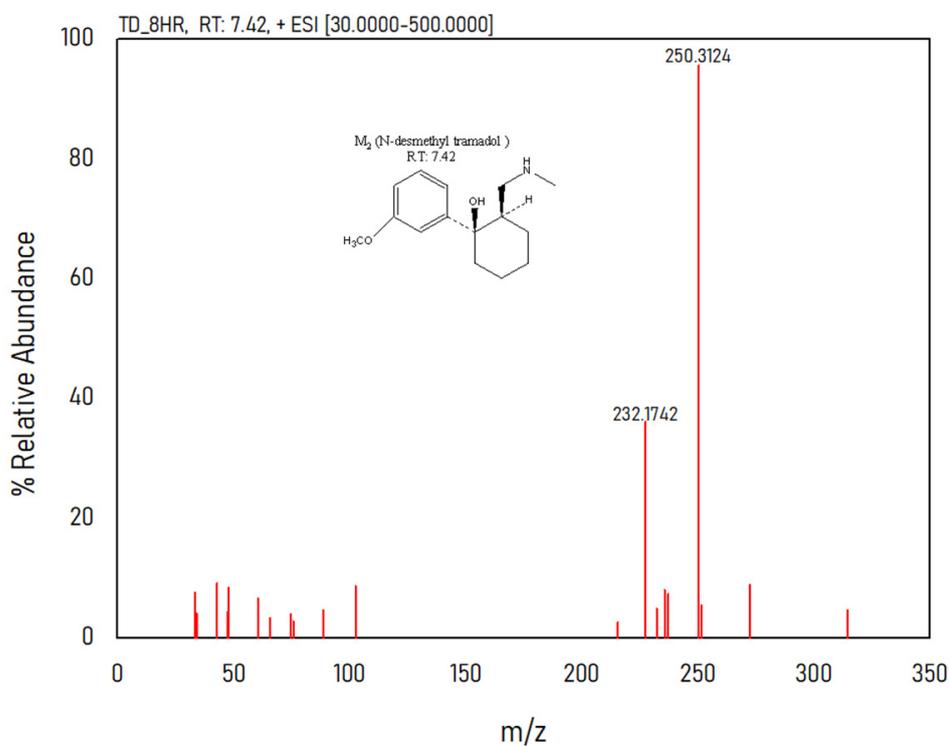
**Figure 2.** (A) Mass spectrum and (B) the TIC chromatogram of the urine extract (collected from a volunteer) of a TD and phase I metabolites (orange) and blank urine extract (blue) using CH<sub>3</sub>OH:H<sub>2</sub>O (35:65) with 0.2% HCOOH as the mobile phase at a flow rate of 0.5 mL/min using a HyPURITY C18 column.

### 3.2.2. Optimization of Mass Conditions

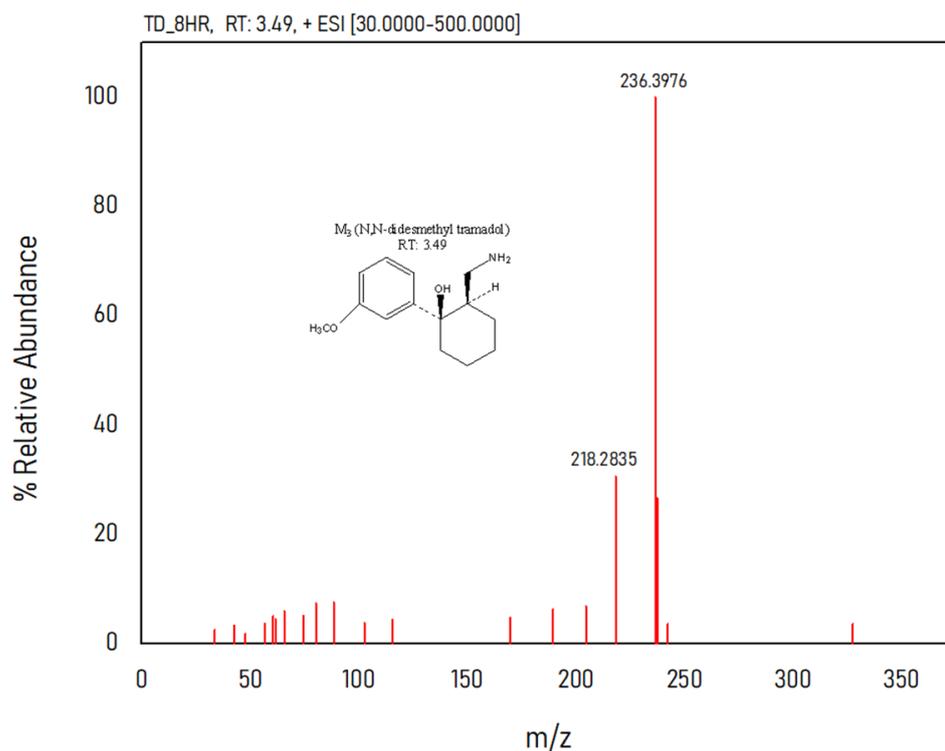
During MS/MS optimization, the compound was initially directly introduced into the MS detector through ESI ionization to achieve optimal MS conditions. Spray voltage, auxiliary gas pressure, vaporizer temperature, sheath gas pressure, and capillary temperature were all optimized to yield protonated pseudo-molecular ions of TD and metabolites. Examining the peak response of fragment ions to determine the appropriate collision energy. When the collision energy was 10 eV, the mass spectra of product ions (Figures 2–7) revealed that TD had a dominant fragment ion peak at  $m/z$  58. We optimized the MS/MS parameters and reported findings.



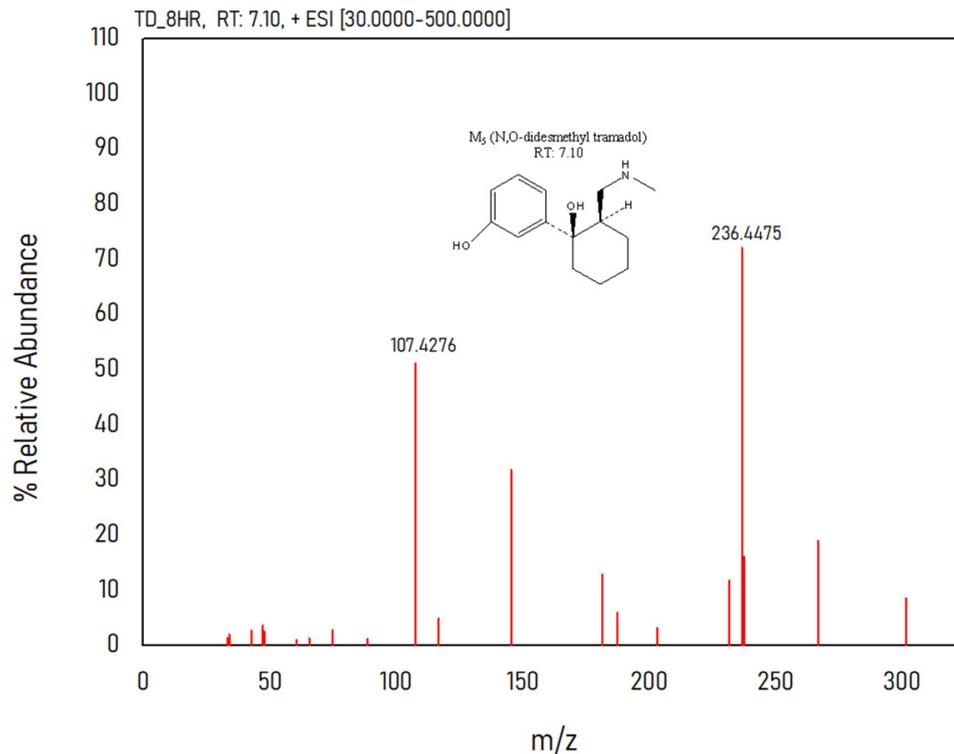
**Figure 3.** Mass spectrum of urine extract at RT: 2.91,  $m/z$  250.3758 corresponding to the  $[M + H]^+$  ion of O-desmethyl TD (M1/ODMT).



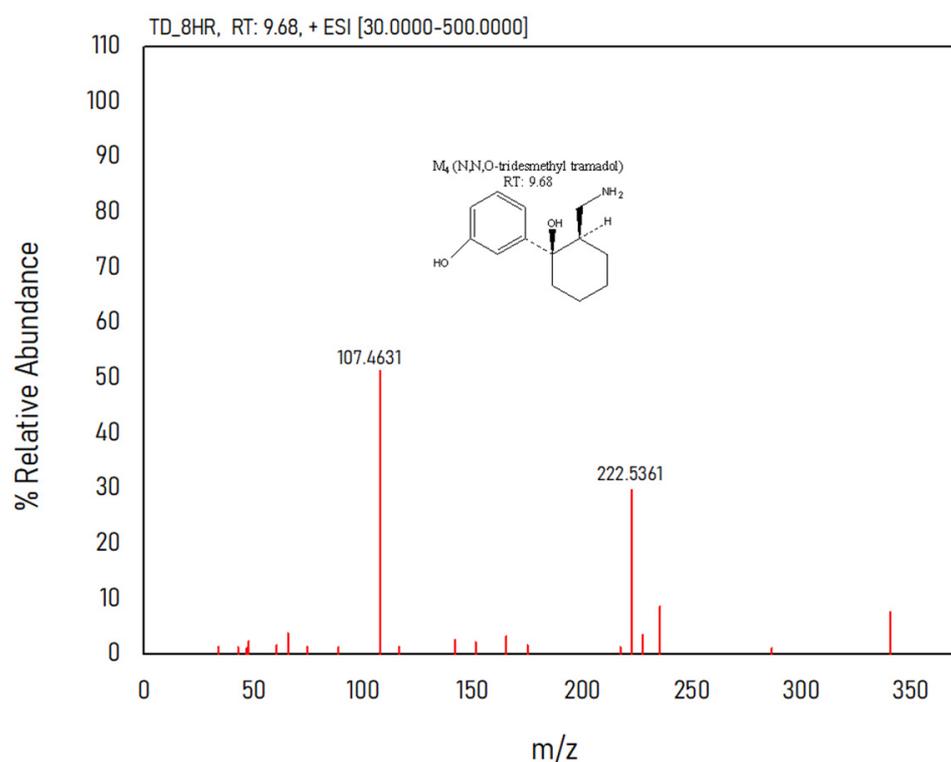
**Figure 4.** Mass spectrum of urine extract at RT: 7.42,  $m/z$  250.3124 corresponding to the  $[M + H]^+$  ion of N-desmethyl TD (M2/NDMT).



**Figure 5.** Mass spectrum of urine extract at RT: 3.49,  $m/z$  236.3976 corresponding to the  $[M + H]^+$  ion of N, N-desmethyl TD (M3/NDT).



**Figure 6.** Mass spectrum of urine extract at RT: 7.10,  $m/z$  236.4475 corresponding to the  $[M + H]^+$  ion of N, N-desmethyl TD (M5).



**Figure 7.** Mass spectrum of urine extract at RT: 9.68,  $m/z$  222.5361 corresponding to the  $[M + H]^+$  ion of N,N,O-tridesmethyl TD (M<sub>4</sub>).

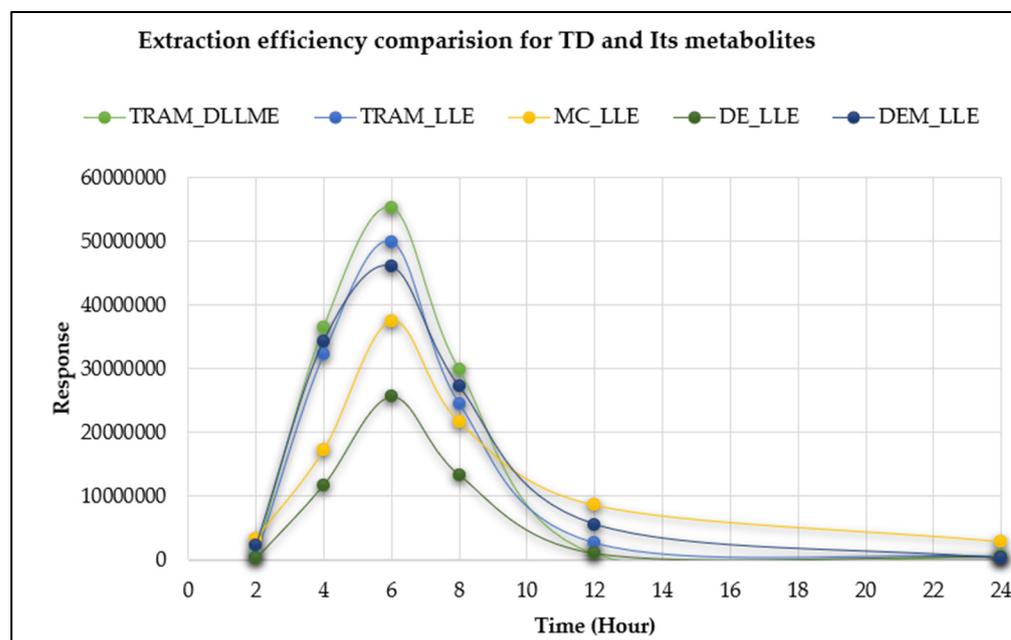
### 3.2.3. Optimization of the Extraction Procedure

Before the LC-MS analysis of complex biological matrices, sample clean-up, whether via liquid–liquid extraction (LLE), dispersive liquid–liquid microextraction (DLLME), or solid phase extraction (SPE), is still suggested. Most techniques published for TD in biofluid determination used LLE to clean up biological samples. As per the previous studies, the DLLME and LLE are the most frequently used extraction procedures for extracting drugs from the urine matrix [26].

Both methods have been performed to accelerate extraction efficiency, as depicted in Figure 8. Thus, various combinations of dispersion and extraction solvents have been used, including chloroform (C), methanol (M), diethyl ether (DE), ethyl acetate (EA), etc. To conduct sample separation using DLLME, specific prerequisites must be achieved. The dispersing solvent must be completely soluble in water. Acetonitrile, acetone, and methanol are commonly utilized for this purpose. The solvent must be extracted to achieve a few characteristics, and it must be able to extract analytes. Additionally, it must be soluble in the dispersion solvent and sparingly soluble in water. Selecting the extracting and dispersing solvents carefully is necessary to achieve a significant enrichment factor. TD and its metabolites were extracted from human urine using a variety of procedures. Multiple organic solvents, such as diethyl ether, dichloromethane, tert-butyl methyl ether, and ethyl acetate, were used to achieve efficient extraction with the DLLME method. Extraction solvent (ethyl acetate and chloroform in the ratio of 1:3) and dispersion agent (methanol) were combined to create the best extraction method at a ratio of 1:2 for 10 min, and the extraction tubes were centrifuged at 4500 rpm. Using this procedure, the efficient extraction of TD along with the maximum number of metabolites was achieved.

In the previously reported extraction procedures, DLLME, LLE, and SPE were used to extract TD with great efficiency but not the metabolites of TD. The DLLME approach for determining TD in urine samples was compared to previously described methods. The suggested approach has a lower LOQ, a better linear range, higher relative recoveries, and a broader range of analytes identified than earlier studies, including phase I and phase II

metabolites. Additionally, DLLME’s extraction process takes less time overall, and there are no labor-intensive or prolonged steps in this process.



**Figure 8.** Represents the extraction efficiency of TD comparison for better recovery rate in the urine sample, which illustrates that DLLME has better extraction efficiency over LLE.

### 3.2.4. Method Validation

Selectivity and specificity are the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in plasma samples. The separation of TD and its metabolites through the utilization of the experimental parameters is described in Table 1.

**Table 1.** Represents the identified and separated phase 1 metabolite of TD found in the urine sample.

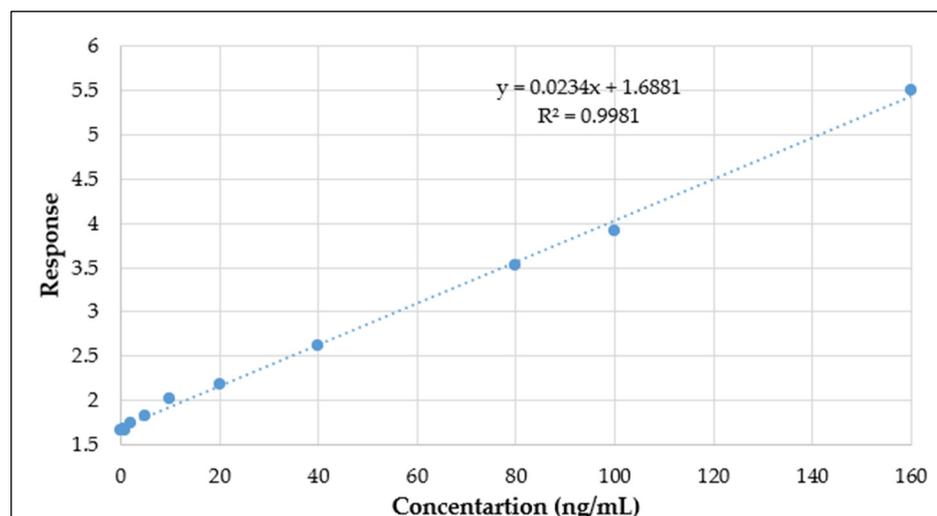
Analyte	Metabolite	<i>m/z</i>	Retention Time
M0	TD	264.4032	5.68
M1	O-desmethyl TD	250.3758	2.91
M2	N-desmethyl TD	250.3124	7.42
M3	N,N-didesmethyl TD	236.3976	3.49
M4	N,N,O-tridesmethyl TD	222.5361	9.68
M5	N,O-didesmethyl TD	236.4475	7.1

The selectivity of the methodology was demonstrated by the absence of an endogenous MS response and/or peak at analyte retention times. The chromatograms of reference urine and urine-spiked samples with drugs demonstrated no endogenous interference during the retention time of the peak of interest, which validated selectivity. TD (M0), M1, M2, M3, M4, and M5 retention times were 5.68, 2.91, 7.42, 3.49, 9.68, and 7.1 min, respectively, represented in Figures 2–7.

Using a weighted least squares approach, it was observed that the calibration curves for the urine test created with a peak area ratio (*y*) of TD versus drug concentration were linear across the concentration range of 0.1–160 ng/mL. The linear regression equations of the calibration curves are summarized in Table 2. The standard curve correlation coefficient was 0.9981 (Figure 9).

**Table 2.** Illustrates the linearity of TD in human urine.

Parameter	TD
Linearity (ng/mL)	0.1–160
Regression equation	$y = 0.0234x + 1.6881$
Standard deviation of slope	0.023387819
Standard deviation of intercept	1.68806942
Correlation coefficient	0.9981
Limit of detection (ng/mL)	0.92
Limit of quantification (ng/mL)	2.7



**Figure 9.** Represents the calibration curve of TD in human urine linearity over the concentration range.

Table 3 summarizes the findings from the method’s validation on the urine matrix. Urine samples were analyzed at three different concentrations six times daily to determine repeatability. The same urine samples were examined once per day for three days to determine the intermediate precision and accuracy. The approach was precise and accurate, and accuracy at three concentrations ranged from 94.85 to 110.25%, and within- and between-day RSD ranged from 1.15 to 3.11% for TD. The absolute recovery of TD for three different concentrations was 94.65, 93.51, and 96.81% for 5, 50, and 150 ng/mL, respectively.

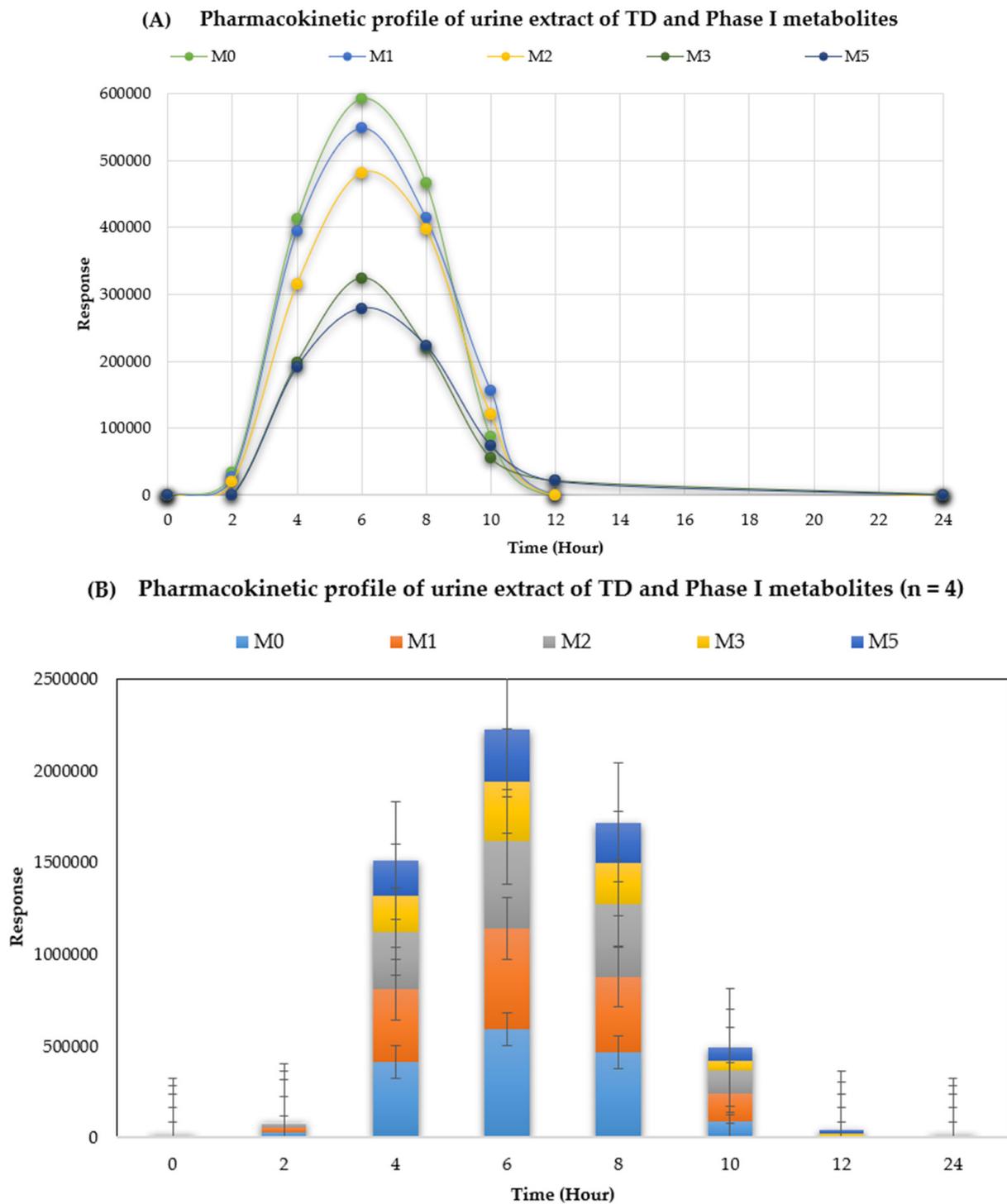
**Table 3.** Shows intra-day and inter-day accuracy, precision, and recovery in human urine samples ( $n = 4$ ).

Analyte	Concentration (ng/mL)	Day 1		Day 2		Day 3		Inter-Day RSD %	Recovery (%)
		RSD %	Accuracy %	RSD %	Accuracy %	RSD %	Accuracy %		
TD	5	1.98	99.65	1.85	98.52	1.90	95.56	3.92	96.29
	50	2.05	99.92	2.98	99.82	2.08	96.68	2.32	96.91
	150	3.58	100.21	2.05	97.99	1.92	97.60	2.92	94.31

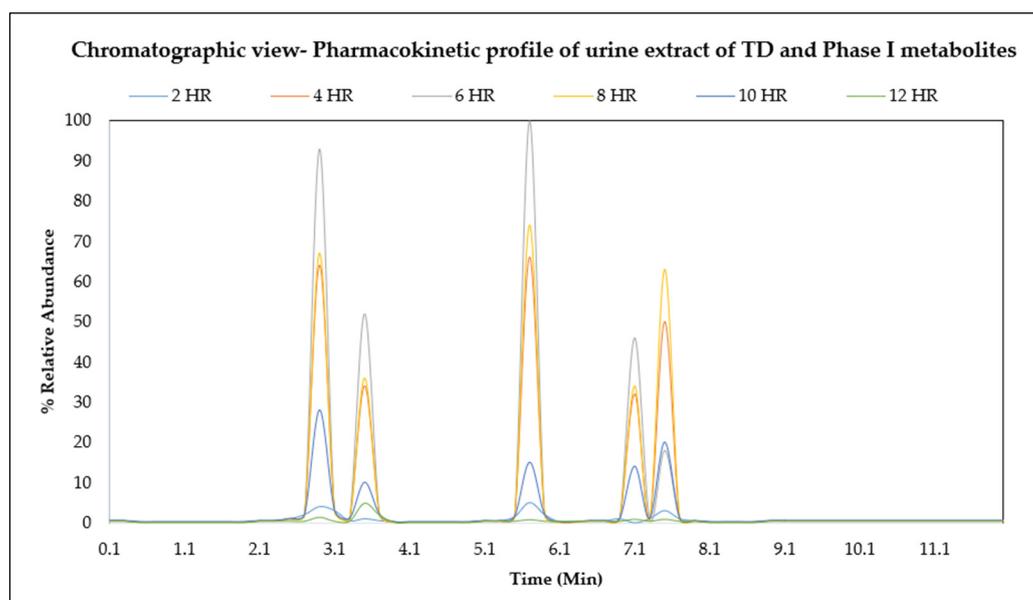
This is, to the best of our knowledge, the first investigation of a widely accepted method for the determination of TD and its phase I and phase II metabolites in urine samples. This was accomplished using ESI-LC-MS/MS, and the results demonstrated that TD distribution and presence in the urine samples were rapidly distinguished from other compounds. ESI-LC-MS/MS was used to determine the pharmacokinetic and elimination pattern of TD and its phase I metabolites after oral administration in human urine.

The two primary and three minor phase I metabolites of TD, M1, M2, M3, M4, and M5, as well as the pharmacokinetic profiles, were all studied in the current paper. Human

urine samples were also taken from the individuals for the study. The TD pharmacokinetic profiles and metabolism in human urine are shown in Figures 10 and 11. The methodology utilized in this work is to separate and identify phase one metabolites. Similar but generated in variable concentrations are the primary phase I metabolites M1 and M2, M3 and M5, and M4. Nevertheless, prior research showed that M1 produced far too little in horse species, with M2 being the predominant metabolite produced. M1 has been found to be the major metabolite in humans as compared to M2 [27].



**Figure 10.** (A) The pharmacokinetic profile of the urine extracts of TD and phase I metabolites. (B) The average pharmacokinetic profile of the urine extracts of volunteers ( $n = 4$ ).



**Figure 11.** The TIC chromatographic view of the pharmacokinetic profile of urine extracts of TD and phase I metabolites.

Thus, it is probable that in vivo M1 is responsible for TD’s “opioid-derived analgesic effect,” making it a suitable experimental model for studying the biological impacts of drug misuse and TD’s neurotoxicity. TD and phase I metabolites were detected in human urine samples up to 24 h later using this method’s sensitivity. Figure 11 shows the patient’s cumulative urine excretion curve for TD and phase I metabolites after taking a single Tramadol tablet (50 mg TD HCl). Even though we only studied one participant, our findings are consistent with the literature [28].

The developed method validated TD in the urine sample collected from the four volunteers at various times. Along with the validation of TD in the urine sample, various phase I and phase II metabolites were identified with separation in the run time. The validated method was applied to the pharmacokinetic study of TD after 50 mg TD to five individuals. The sensitivity and specificity of the method were found to be sufficient for accurately characterizing the pharmacokinetics of TD in this study.

Since most of the previously developed approaches were concentrated on the identification of TD, ODT, and NDT, our methodology was developed to identify and separate a wider range of TD metabolites from the single dosage of TD from urine samples (Table 4).

**Table 4.** Comparison of the proposed method with other methods for the analysis of tramadol and metabolites.

Ref.	Method	Column	Mobile Phase	Sample Matrix	Extraction Recovery (%)	Extraction Procedure	Analytes Identified	Correlation Coefficient
[7]	ESI-LC-MS/MS	Phenomenex Luna® Omega 1.6 µm polar C18	CH <sub>3</sub> OH/H <sub>2</sub> O (70:30, v/v), 0.1% (v/v) formic acid	Human Plasma and Urine	86.51–93.38	LLE	TD	0.9998
[9]	HPLC	Chromolith™ Performance RP-18e	CH <sub>3</sub> OH: H <sub>2</sub> O (13:87, v/v) adjusted to pH 2.5 by phosphoric acid	Human Plasma	-	LLE	TD, M1, and M2	0.997
[17]	HPLC	Chromolith™ Performance RP-18e	CH <sub>3</sub> OH: H <sub>2</sub> O (19:81, v/v) adjusted to pH 2.5 by phosphoric acid	Human plasma, saliva, and urine	86.2–92.9	LLE	TD, M1, M2, and M5	0.996

**Table 4.** *Cont.*

Ref.	Method	Column	Mobile Phase	Sample Matrix	Extraction Recovery (%)	Extraction Procedure	Analytes Identified	Correlation Coefficient
[29]	LC-MS/MS	Aquasil C18	1.0 mL, 1 M ammonium trifluoroacetate, and 0.100 mL of HCOOH in CH <sub>3</sub> OH: H <sub>2</sub> O (70:30, v/v) a	Human Plasma	93.6–98	LLE	TD and M1	0.9989
[27]	HPLC-FL, HPLC-MS/MS	Phenomenex Luna <sup>®</sup> C18 ODS2	CH <sub>3</sub> OH plus HCOOH (0.1%): buffer (ammonium acetate, 5 mM, pH 4.5) (30:70, v/v)	Dog urine	82	-	TD, M1, M2, and M5	0.999
Current Study	ESI-LC-MS/MS	HyPRITY C18 column	CH <sub>3</sub> OH: H <sub>2</sub> O (35:65) with 0.2% HCOOH	Human Urine	94.31–96.91	DLLME	TD, M1, M2, M3, M4, M5, (Phase II metabolites)	0.9981

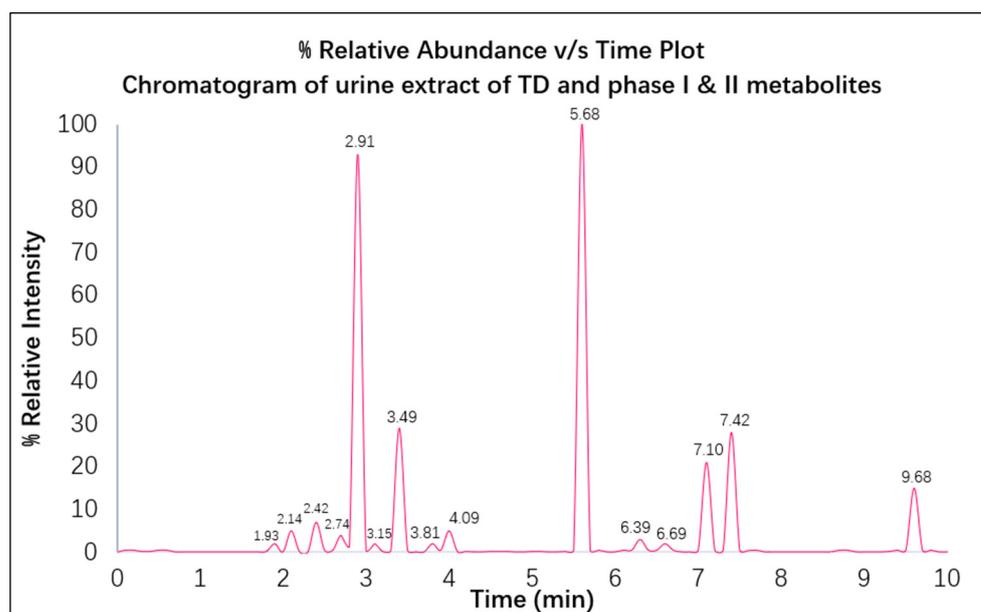
### 3.3. Application Method

The method has been validated through TD analysis and its phase I and II metabolites in urine, followed by a single oral dose of 50 mg TD. As a result of liver metabolism, the study found 26 metabolites for TD, including 14 from phase I (M1 to M11 and M31 to M33) and 12 from phase II (namely 7 glucuronides—M12 to 18—and five sulfonates—M19 to 23). However, just 23 of the 26 metabolites that TD produces after oral treatment were found in the urine [25,30,31]. M31 to M33 were discovered solely in liver microsomes at the same time. M12, M19 through M23, and M33 were not found in mice or dogs among these 26 human metabolites. Only the metabolites O-desmethyl TD (M1) and O, N-didesmethyl TD (M5) are pharmacologically active due to the liver’s metabolism of TD. M1 is cytochrome P450 2D6’s end product of O-demethylation (CYP2D6) [6,32,33].

A total of 14 TD metabolites including five phase 1 (M1-M5) and nine phase II metabolites, three sulfo- and glucurono-conjugated metabolites, three oxidative TD derivatives, and three sulfo-conjugated TD metabolites identified in healthy individuals’ urine samples, were extensively characterized in our study. Additionally, our analysis was the first to use the achiral technology based on ESI-LC-MS/MS to detect 14 TD metabolites, encompassing phase I and II. Figure 12 shows the average excretory profile of TD and phase I and II metabolites in the urine. Table 5 shows the phase II metabolites that were identified (Figures S1–S9).

**Table 5.** Illustrates the phase II metabolites detected in the urine sample (*n* = 4).

Metabolic Pathway	Name	Metabolite	<i>m/z</i>	RT (min)
Sulfo- and glucurono-conjugated TD metabolites	M13	O-desmethyl TD glucuronide	426.3138	6.69
	M16	OH-TD glucuronide	456.4084	6.39
	M15	N,O-desmethyl TD glucuronide	412.1774	3.81
oxidative TD derivatives	M6	OH-TD	280.3856	2.74
	M8	OH-didesmethyl TD	252.2158	1.93
	M32	OH-O-desmethyl TD	266.4093	4.09
Sulfo-conjugated TD metabolites	M23	OH-TD Sulphate	360.4492	3.15
	M21	N, N, O-desmethyl TD sulfite	302.4325	2.14
	M22	N, O-desmethyl TD sulfite	316.4469	2.42



**Figure 12.** Represents the TIC chromatographic view of the separation of TD and phase I and phase II metabolites extracted from urine extract.

#### 4. Conclusions

Globally, the use and abuse of prescription and regulated drugs are rising. Drug overuse may vary from nation to nation but a few substances cross their boundaries via misused technology, the growth of the darknet, and the Internet's role in connecting the world. One of these drugs is TD, a narcotic recently discovered by enforcement officials to be widely abused worldwide. As a result, analytical approaches are increasingly being used to identify pharmaceutical preparations and toxicological samples, including blood, urine, saliva, and hair. This study uses an achiral column where the methodology based on LC-MS/MS with ESI has been developed and validated for detecting TD and its phase I and phase II metabolites in human urine. A precise and succinct DLLME method was used for samples. For four volunteer urine samples, the DLLME method provides strong repeatability and increased recovery in a short period of time. Analyzing TD and its metabolites in the urine matrix is thus practical for forensic and therapeutic drug-monitoring studies. In conclusion, the current work created a straightforward, affordable, reliable, and wide throughput analytical approach for estimating TD and its major metabolites simultaneously in human urine. The pharmacokinetics and metabolism data given in the present study provide information for the design of possible future research disorders evaluating drug mechanism and neurotoxicity and for the effective application screening of TD.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/separations10060365/s1>, Figure S1: MS/MS of urine extract  $m/z$  426.3138 corresponding to  $[M + H]^+$  ion of O-desmethyl TD Glucuronide (M13); Figure S2: MS/MS of urine extract  $m/z$  456.4048 corresponding to  $[M + H]^+$  ion of OH TD Glucuronide (M16); Figure S3: MS/MS of urine extract  $m/z$  412.1174 corresponding to  $[M + H]^+$  ion of N,O-desmethyl TD Glucuronide (M15); Figure S4: MS/MS of urine extract  $m/z$  280.3817 corresponding to  $[M + H]^+$  ion of OH TD (M6); Figure S5: MS/MS of urine extract  $m/z$  252.2158 corresponding to  $[M + H]^+$  ion of OH-didesmethyl TD (M8); Figure S6: MS/MS of urine extract  $m/z$  266.4093 corresponding to  $[M + H]^+$  ion of OH-O-desmethyl TD (M32); Figure S7: MS/MS of urine extract  $m/z$  360.4492 corresponding to  $[M + H]^+$  ion of OH-TD Sulphate (M23); Figure S8: MS/MS of urine extract  $m/z$  302.4325 corresponding to  $[M + H]^+$  ion of N,N,O-desmethyl TD Sulphate (M21); Figure S9: MS/MS of urine extract  $m/z$  316.4469 corresponding to  $[M + H]^+$  ion of N,O-desmethyl TD Sulphate (M22).

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