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In-Vitro Modified QuEChERS Based Analytical Method for Simultaneous Detection of Antidepressant Drugs from Biological Matrices Using LC-MS/MS

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Abstract: Though antidepressants are a group of psychoactive drugs recommended for prescription use, their abuse is uncommon. With the advent of analytical techniques, several methods have been developed for extracting antidepressants from complex biological matrices. However, these developed methods are perfunctory and require a better protocol for evaluation. In this study, a modified QuEChERS (quick, easy, cheap, efficient, robust, and safer) method was performed to determine the optimal conditions to quantify three categories of antidepressants by Liquid chromatography with tandem mass spectrometry from saliva, urine, and gastric fluid samples. The injection volume of the sample solution kept at 10 uL, and the temperature of the analytical column was remained at 40°C, with variable concentrations of 5, 10, 20, 50,100, and 200 ng/mL. The limit of quantification ranged from 07.35-12.08 ng/mL, and the limit of quantification from 22.27-38.31 ng/mL for all the drugs. We hereby report the successful extraction of Tramadol, Tianeptine, and Mirtazapine for the first time with a higher recovery rate (> 72%) in biological matrices. This approach can be useful for extracting Tramadol, Tianeptine, and Mirtazapine from various biological matrices as it requires a considerably low amount of solvent utilized, thereby reducing the generated waste.

Keywords: antidepressants; QuEChERS; LC-MS/MS; quantification; simulated matrices.

1. Introduction

Depression is stated as a major depressive disorder which is the most commonly occurring public health problem worldwide [1]. Antidepressants (ADs) are a class of psychoactive drugs used as a therapy for major depressive disorders since the advent of Imipramine in 1952. Although such drugs are prominently used in clinical environments, some have been associated with unintentional deaths and suicide [2]. They are categorized into four groups that are – Selective serotonin reuptake inhibitors (SSRIs), selective serotonin and norepinephrine reuptake inhibitors (SNRIs), Tricyclic antidepressants (TCAs), Monoamine oxidase inhibitor antidepressants (MAOIs), and atypical antidepressants. ADs can assist with depression and anxiety but also have some adverse side effects [3]. TCAs & SNRIs

consumption induces cardiac arrhythmias, respiratory depression, metabolic acidosis, convulsions, and coma, rendering it a common cause of suicidal death [4].

Among SNRIs, Tramadol (TMD), or (1R, 2R)-2-(dimethyl amino) methyl)-1-(3-methoxyphenyl) cyclohexane-1-ol, as shown in Figure 1, is regarded as powerful ADs with a μ-receptor agonist used to ease moderate to severe pain[5]. Whereas Tianeptine (TNP) or 7-[(3-chloro-6-methyl-5, 5-dioxo-11H-benzo[c] [2, 1] benzothiazepin-11-yl) amino] heptanoic acid as shown in Figure 1 is a new generation TCAs having a selective serotoninergic activity. Since its release in 1988, it has been widely utilized in the treatment of depressive disorders. TNP is presently the second most prescribed AD (after amitriptyline), being prescribed to more than 20% of depression patients in psychiatric clinics[6].

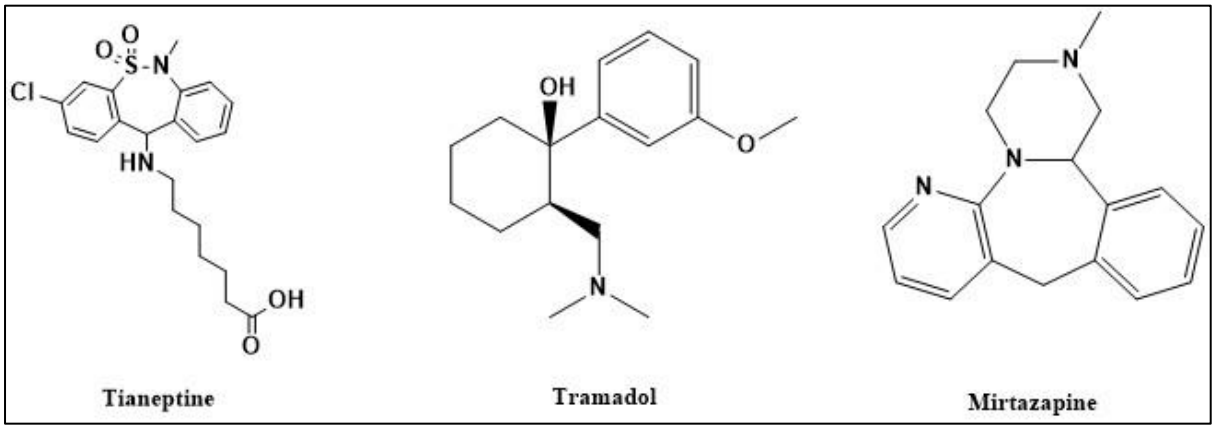

Figure 1. Chemical structure of Tramadol, Tianeptine, and Mirtazapine.

Another category includes Mirtazapine (MTZ) or Remeron as an atypical antidepressant (ATAs) chemically written as 1,2,3,4,10,14b-hexahydro-2-methylpyrazino b [2,1-a] pyrido [2,3-c] benzazepine which has the molecular formulae of $C_{17}H_{19}N_3$ and molecular weight as 265.36 as illustrated in Figure 1. MTZ is a component of the piperazinoazepine mixture which is equally effective at treating chronic depression as the TCAs amitriptyline and clomipramine. The ADs effects of MTZ are attributed to increased serotonergic and noradrenergic receptors in the CNS [7]. The noradrenergic action of MTZ is mediated through the blockade of inhibitory presynaptic á2-autoreceptors. This inhibition causes a more rapid release of norepinephrine into the synapses and increases its bioavailability postsynaptically. MTZ does not, therefore, inhibit norepinephrine reuptake. MTZ also inhibits the á2-heteroreceptors of the serotonergic nerve terminals, increasing the production of serotonin [8]. MTZ is absorbed rapidly after a single dose and achieves its peak plasma concentration (Cmax) in 1 to 2.1 hours. After a few doses, the Cmax of MTZ is reached in 1.1 to 2.9 hours. The nonspecific and reversible affinity of MTZ for plasma proteins is 85%. Its absolute bioavailability is just about 50% because of the gastrointestinal tract and hepatic first-pass metabolism. The liver, urine, and gastric lavage significantly metabolize MTZ, which has an elimination half-life of 20 to 40 hours [9]

Various sophisticated analytical techniques have been developed to examine these drugs in clinical and forensic toxicological settings. But all the developed techniques had high sample consumption, low recovery rate, high matrix effect, or complex analytical methods [10–13]. Saliva, urine, and gastric fluid are the most prevalent specimens in postmortem toxicology after whole blood because they strongly associate with biological (therapeutic or toxic) effects. Blood is the most important fluid, which establishes a direct link between concentration and

effect. However, it is also known that various forensically important drugs are excreted in other biological matrices, such as urine, saliva, and gastric juice secreted in oral fluids, which can help correlate the quantity in matrices. Moreover, even in patients who have defied the odds, urine and saliva can be obtained easily with non-invasive methods.

Several traditional sample extraction protocols, such as liquid-liquid extraction (LLE), solid-phase extraction (SSE), solid-liquid extraction (SLE), and microextraction for biological sample preparation, have a certain limitations, including poor analytical accuracy, significant solvent use, and difficult separation method, and labor-intensive and exhaustive methodology[14–[16]. QuEChERS is another adaptable method for extracting drugs from biological samples that have gained popularity in forensic toxicology. Anastassiades *et al*. were the first to use it to investigate pesticides in vegetables, but investigations using human samples have also yielded positive results[17,18]. There are two fundamental steps: an extraction helped by salting out and a clean-up using dispersive solid-phase extraction (dSPE).

In previous literature, hyphenated techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are commonly used to identify drugs and toxins[2,19–21]. ADs are generally polar compounds, and they are frequently detected in bodily fluids using an LC-MS/MS. LC-MS/MS is very useful in regular drug tests because of its analytical robustness; with the inclusion of MS, It is also a potent instrument for intensifying the signal-to-noise ratio to target potential analytes in biological matrices at the nanoscale level. This technology has several other advantages, including sensitivity, selectivity, quick analysis time, and a relatively high throughput allowing multiple compounds to be analyzed with a single method. Consequently, the LC-MS/MS is deemed useful and effective for analyzing, identifying, and quantifying a wide range of drugs, including ADs [22,23].

Suicide attempts using a life-threatening overdose of drugs are not uncommon among depressive disorder patients. Toxicity and overdose cases of TNP, TMD, and MTZ are reported in many previous works of literature [24–28]. In both therapeutic and forensic contexts, detecting illicit substances in biological matrices is critical. Due to the complexity of the matrices, direct detection of such samples is frequently difficult [29]. For example, salts creatinine, urea, proteins, and other foreign substances present in urine, saliva, and gastric fluid might obstruct the analysis and yield a higher matrix effect [30,31]. To overcome such factors simulated matrices are used, other than *in vitro* analysis, simulated matrices also shows the similar result and better accuracy than biological matrices [32–35].

This study has been carried out to simultaneously extract and quantify three ADs (Tianeptine, Tramadol, and Mirtazapine) from simulated biological samples. TNP TMD and MTZ were extracted by the QuEChERS method followed by LC-MS/MS for quantification. The study was conducted per the Scientific working group of forensic toxicology (SWGTOX) guidelines[36].

2. Materials and Methods

2.1. Chemical and reagents.

All chemicals and reagents used in this study are of analytical grade. Standard TNP, TMD, and MTZ were obtained from SIMA LABS (Sophisticated Industrial Materials Analytic Labs Pvt. Ltd.), New Delhi, India. For HPLC (High-Performance Liquid Chromatography), methanol, water, formic acid, and ammonium formate solution were procured from

Sigma Aldrich, St. Louis, MO, USA. For dispersive solid-phase extraction (d-SPE): EN QuEChERS salt pouch (Agilent: 5982-0650 Agilent Technologies, Inc., Santa Clara, CA, USA), was accompanied by sodium citrate tribasic dihydrate (1 g) and sodium citrate dibasic sesquihydrate (0.5 g). Anhydrous magnesium sulfate, sodium chloride, sodium acetate, and primary secondary amine (PSA) were also obtained from Sigma Aldrich, St. Louis, MO, USA.

2.2. Quality controls and internal standards.

The standard solutions (stock, work, and calibration) were synthesized in methanol at 01 mg/L and stored at -20 °C. Working saturated solutions of drugs were synthesized by diluting in methanol depending on their concentrations in simulated saliva, urine, and gastric fluid at their therapeutic levels. Working solutions of the mix standards were synthesized for calibration by serially diluting the stock solution at six concentration levels: 5, 10, 20, 50, 100, and 200 ng mL^{-1} for all drugs.

2.3. Preparation of simulated matrices.

An artificial eight-component urine sample was prepared in accordance with Stolarz *et al*. (2005) protocol [37]. Simulated urine containing about 17 g/L of urea (to simplify the organic composition, only urea was utilized as an organic component), chlorine (9.60 g/L), sodium (5.40 g/L), sulfate (1.35 g/L), magnesium (0.65 g/L), calcium (0.20 g/L), and potassium (0.20 g/L), was added in distilled water (volume 1.0 L) at pH 6.0. Simulated saliva was synthesized according to Pietrzyńska *et al*. (2017) by dissolving the entire contents in distilled water to make a total volume of 1L [38]. For gastric fluid, the artificial gastric juice was made with 0.03 M aq. sodium chloride, 0.084 M aq. hydrochloric acid, and 0.32 percent (w/v) pepsin, according to the United States Pharmacopeia protocol[39].

2.4. QuEChERS extraction.

QuEChERS extraction was optimized by implementing the two strategies: Step 1 (partitioning): 10 mL Milli Q water was added to a 50 mL centrifuge tube having 5ml of homogeneous simulated urine, saliva, and gastric fluid sample spiked with different concentrations 5, 10, 20, 50, 100, 200 ng/mL of TMD and TNP. To this mixture, 10 mL of methanol was added, which acts as a diluent. This mixture is kept again in a wrist action shaker for 10 mins to homogenize the sample. To this homogenized sample, EN QuEChERS salt pouch (was added in 15 mL of the centrifuge tube and then mixed it using a vortex shaker for 1 more min., then the sample was centrifuged at 6000 rpm for 10 minutes. at 2-8 °C using a refrigerated centrifuge. From the final solution, 6 mL of the supernatant layer was taken out into a 15 mL centrifuge tube. Step 2 (dSPE): The obtained mixture was cleaned up with 500 mg of magnesium sulfate to remove moisture and water molecules from the sample, followed by 250 mg of primary and secondary amine (PSA) to make the compound simpler. Then the sample was vortexed for 2 mins and centrifuged at 6000 rpm for 6 mins at 2-8 ° C. The supernatant was poured into a tube containing 150 mg of $MgSO_4$, then vortexed and centrifuged for 5 minutes at 12.000 rpm. An aliquot of 200 uL of the final extract was relocated to a different vial for all clean-ups, and 10 uL from all samples was injected directly into the LC-MS/MS apparatus.

2.5. LC-MS/MS condition.

LC-MS/MS (Agilent 6470B, Agilent Technologies, Inc., Santa Clara, CA, USA) analysis was carried out in positive-ion mode, with the following parameters for precursor ion scans: 100-500 m/z; 0.3-2.0 V ramping collision energy (Smart Frag); 4 amu precursor ion isolation width; one MS and one MS/MS spectra analysis. The flow rate was set at 0.4 mL/min, the injection volume of sample solution at 10 uL, and the temperature of an analytical *column Poroshell* 120 with bonded phase EC-C18 (2.7 µm, 3mm x *150 mm)* was kept at 40°C. For the purpose of evaluating and quantifying TMD and TNP, the MS/MS results were determined utilizing the MRM (multiple reaction monitoring) modes, which had their transitions and collision energies tuned.

3. Results and Discussion

3.1. Optimisation of QuEChERS method.

Even though the QuEChERS method has a descriptive and exploratory nature, its efficacy is dependent on a few factors, and the selectivity of the extraction method can be altered. Because of the flexibility in introducing changes to traditional methods and to enhance the extraction process, studies like this are important to assess specific parameters, such as the extraction solvent to be used, sample/solvent ratio, pH, type of agitation, type and quantity of partition salts and cleaning sorbents[40,41].

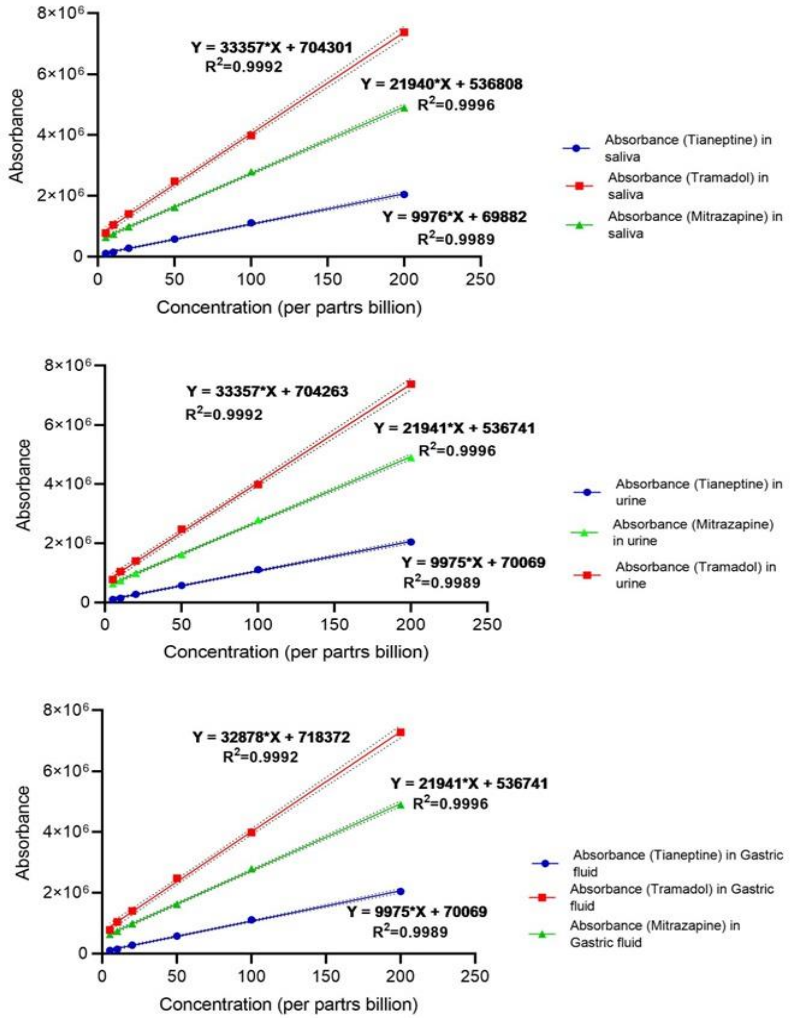

Figure 2. Calibration curve of TNP, TMD, and MTZ in saliva, urine, and gastric fluid.

The initial application of QuEChERS was considered in two steps, and a design experiment reviewed the extraction technique to determine the optimal condition[42]. Methanol was chosen apart of acetonitrile because it offers mild protein precipitation and is miscible with water(18). The d-SPE step considered the removal of co-extracted components, employing sorbent like PSA and a new commercial substance called EMR-lipid, both with the administration of $MgSO_4$ leads to the removal of leftover water or salt residue in the sample [42]. The preliminary assessment was constructed on a comparison of the amplitude and statistical significance of parameters concerning the effects on the response (chromatographic peak regions) that were yielded from the generated normal plots for each ADs. Figure 2 shows representative calibration plots for TNP, TMD, and MTZ in saliva, urine, and gastric fluid samples. The linear plots with a goodness of fit parameter (R^2) ranging from 0 to 1 indicate that the predictions from the regression perfectly fit the data.

Finally, all analytes and their significant impacts were included in the optimization process via "numerical optimization," a method relying on the desirability idea that combines the effects of responses and factors to produce the best final conditions. The following optimal conditions were utilized desire level (98.3%): solvent: methanol; agitation: vortex or homogenizer; sorbent clean-up: PSA + $MgSO_4$ (1:6) (w/w); partitioning salts: Sodium Acetate (NaAc): MgSO4 (1:4) (w/w). Consequently, the DOE-optimized QuEChERS extraction parameters were used in the subsequent technique validation.

3.2. Implementation of LC-MS/MS condition.

The optimal chromatographic and spectrometric conditions for TMD, TNP, and MTZ were obtained by diffusing clean standard solutions directly into the LC-MS/MS system. Each precursor ion was observed first, followed by the identification of two separate product ions for each sample utilizing various collision energy voltages, among the transitions serving as the quantifier ion and the peak with the second-highest intensity serving as the qualifier ion. Then, utilizing multiple reactions monitoring (MRM) transitions and dwell periods, the parameters were automatically modified.

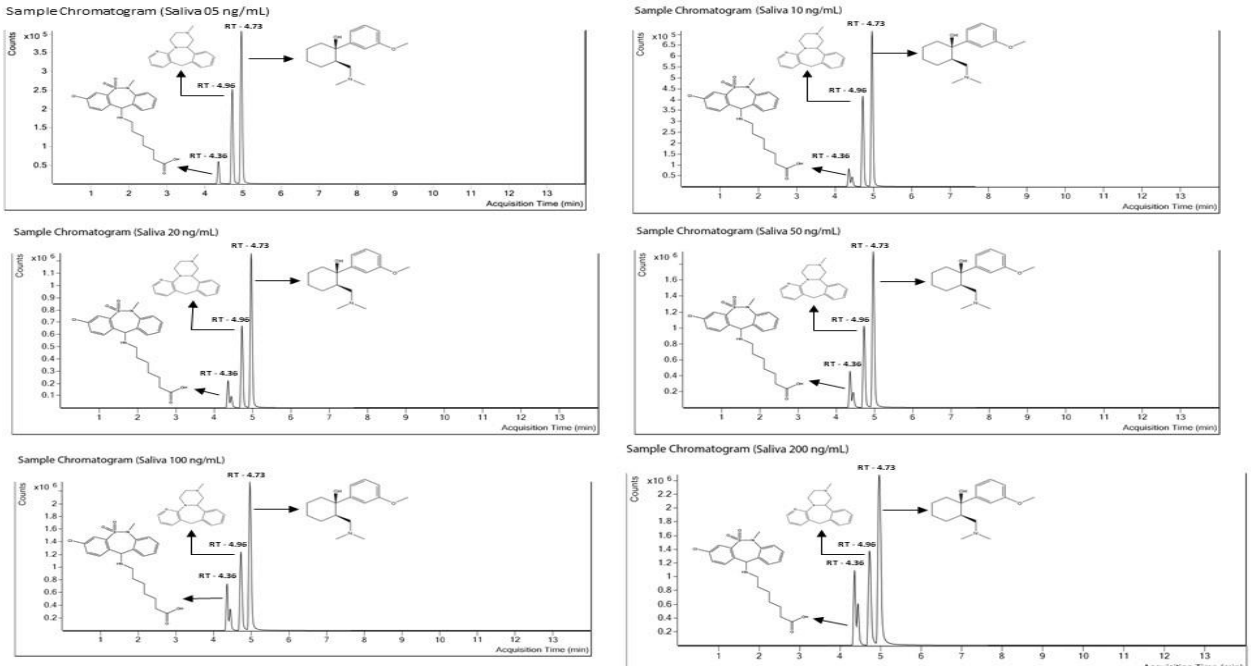

Figure 3. LC-MS/MS chromatograms (acquisition time vs. counts) of TNP, TMD, and MTZ at a concentration of (**a**) 5; (**b**)10; (**c**) 20; (**d**) 50; (**e**) 100 and (**f**) 200 ng/mL in saliva.

Apart from MS conditions, the composition of the mobile phase, such as the concentration and pH of the buffer solution, plays a critical role in enhancing quantification specificity because it impacts the peak shape of the analytes in chromatography and the ionization efficiency of the analytes in MS[45]. 5 mM ammonium acetate significantly decreased the peak width and made their peak more symmetric when compared to water. The signal responses of TMD, TNP, and MTZ were considerably enhanced by formic acid (0.1 %). Methanol outperformed acetonitrile in terms of elution. To obtain constant sensitivity and good retention of TMD, TNP, and MTZ, a mobile phase consisting of methanol: 5 mM ammonium acetate: formic acid (35:65:0.1, v/v/v) was adopted following a series of studies. The retention times of TMD, TNP, and MTZ for urine, saliva, and gastric fluid were found under optimal LC conditions, as shown in Table 1. MS parameters were tuning using a 100.0 ng/mL tuning solution in positive and negative ionization modes. In positive ionization mode, the sensitivity was substantially higher with minimal background noise than in negative mode for both drugs. The precursor/product ion mass transitions were measured at 282→ 292 m/z for TNP, 264.1→ 58.1 m/z for the TMD, and 266.35→195.31 m/z for MTZ. The results of LC-MS/MS shown in Figure 3, Figure 4, and Figure 5 demonstrate the simultaneously obtained response and retention time of all three ADs drugs in simulated saliva, urine, and gastric fluid sample at a concentration of 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL using LC-MS/MS.

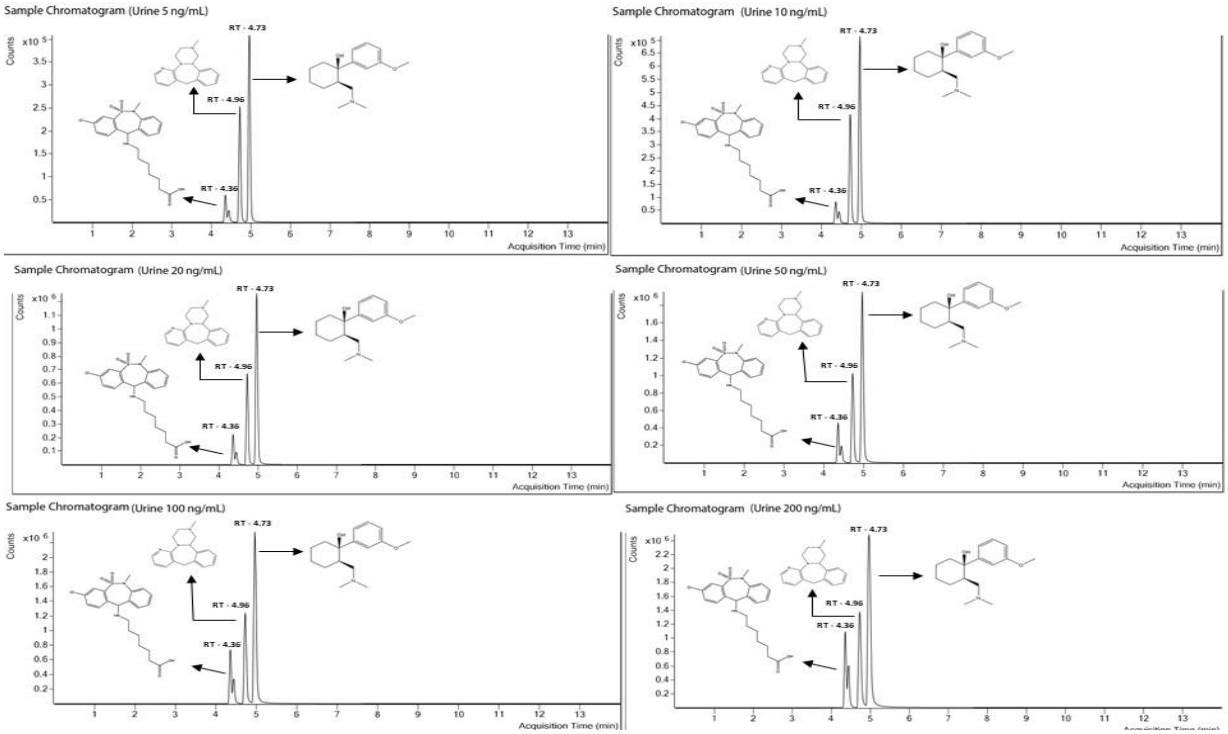

Figure 4. LC-MS/MS chromatograms (acquisition time vs. counts) of TNP, TMD, and MTZ at a concentration of **(a)** 5, **(b)**10, **(c)** 20, **(d)** 50, **(e)** 100 and **(f)** 200 ng/mL in urine.

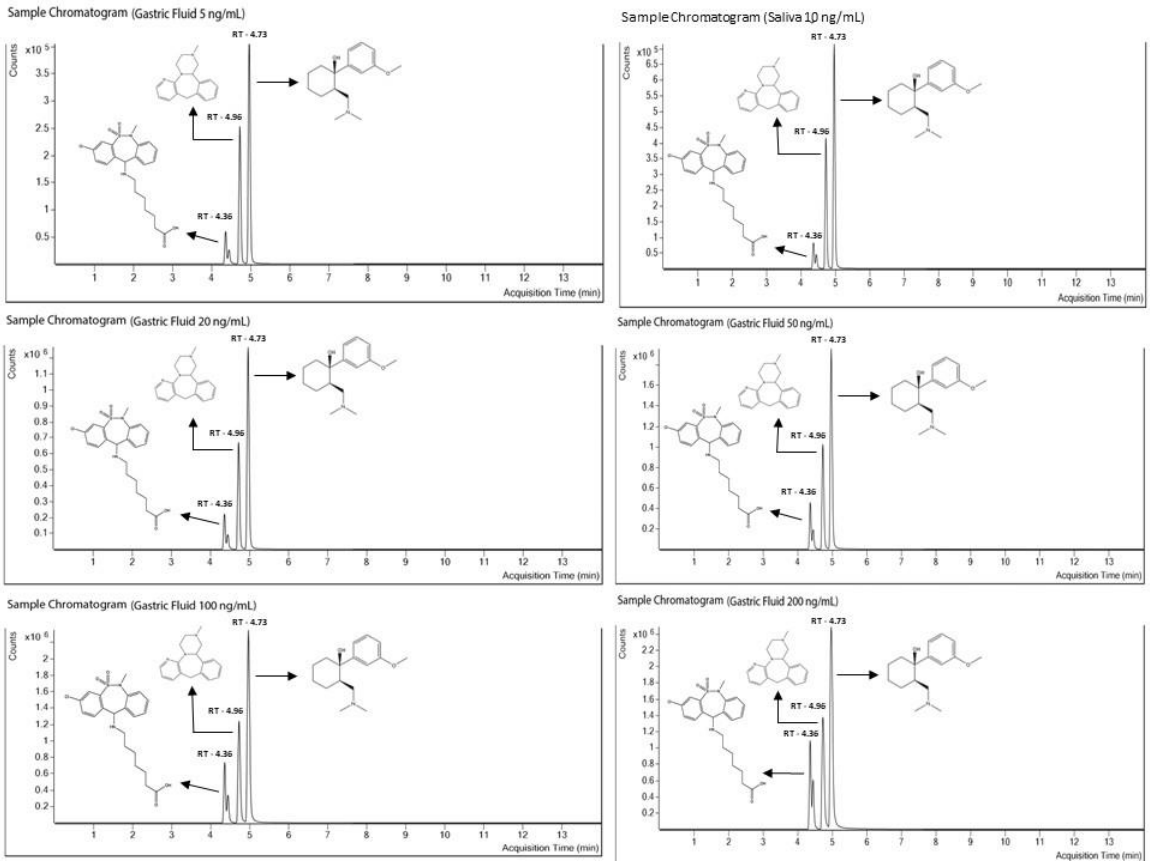

Figure 5. LC-MS/MS chromatograms (acquisition time vs. counts) of TNP, TMD, and MTZ at a concentration of 5, 10, 20, 50, 100, and 200 ng/mL in gastric fluid.

3.3. Evaluation of Calibration model, LOD and LOQ.

The linearity of the presented method was asserted using calibration curves generated from peak-area to standard internal ratios of analytes and six levels of concentration using the statistical software GraphPad Prism V9.0. The study's working range attempted at 10 ng/mL, and the concentration range covered the therapeutic concentration for all drugs. LOD and LOQ of the LC-MS/MS system were assessed using a signal-to-noise ratio of 3:1 and 10:1 and further summarised in Table 1[46]. The results reveal that the calibration range favored a linear curve with $1/x^2$ weighting for most ADs. A linear regression coefficient R^2>0.9999 indicates a reliable level of model adjustment fit.

Table 1. Data of calibration models, LOD, and LOQ for TNP, TMD, and MTZ drug by **QuEChERS** extraction and LC-MS/MS analysis.

Drug	Biological matrices	R^2	Slope	Intercept	LOD (ng/mL)	LOQ (ng/mL)	RSD %	Precursor (m/z)	Product (m/z)	RT
TNP	Saliva	0.9989	9976.4	69881	12.08	36.62	1.23	228	292	4.357
	Urine	0.9989	9975.3	70057	12.03	36.45	1.146	228	292	4.357
	Gastric fluid	0.9989	9975.2	70069	12.64	38.31	1.226	228	292	4.357
TMD	Saliva	0.9992	33357.2	704292.1	10.42	29.53	1.130	264.1	58.1	4.965
	Urine	0.9992	33357.4	704262.9	10.41	29.52	1.145	264.1	58.1	4.965
	Gastric fluid	0.9992	33357.0	704263	10.41	31.54	1.146	264.1	58.1	4.965
MTZ	Saliva	0.9996	21940	19945	7.35	22.27	1.163	266.35	195.31	4.733
	Urine	0.996	21941	19963	7.35	22.29	1.163	266.35	195.31	4.733
	Gastric fluid	0.9996	21941	536741	7.35	22.29	1.163	266.35	195.31	4.733

3.4. Imprecision and recovery.

ANOVA was used to scrutinize imprecision, and a value was obtained as RSD tabulated in Table 1. Imprecision and recovery values provided are consistent with those observed in prior research [41–46], and all samples evaluated met the criteria for imprecision acceptance limits (not exceeding variations of 20%). For the evaluation of recovery, two sets of samples were used: one produced in a blank matrix spiked after extraction (AE), and another spiked in the blank matrix before extraction (BE). The following equations were used to determine the recovery:

$$Recovery\,\% = \left(\frac{BE}{AE}\right) \times 100$$

The recovery percentage for TNP, TMD, and MTZ was obtained as 88 – 114% in simulated saliva and urine sample. For the lowest concentration, such as at 5 ng/mL, QuEChERS proved to be significant in providing recovery of 94.02 – 94.78% in saliva samples, 92.91 – 98.24 % in case of urine and 72.78 – 98.64 % in gastric fluid samples. Obtained concentration and recovery percentage of the analytes are mentioned in Table 2, which are in the ideal range of 70-120 %. Figure 6 also demonstrates the bar graph of obtained recoveries for all the drugs.

Table 2. The recovered concentration of TNP, TMD, and MTZ in saliva, urine, and gastric fluid.

S.no	**Spiked Concentration**	**Saliva**		**Urine**		**Gastric Fluid**	
		Extracted concentration (ng/ml)	Recovery %	Extracted concentration (ng/ml)	Recovery %	Extracted concentration (ng/ml)	Recovery %
Tramadol							
1.	5	4.74	94.78	4.63	92.91	3.63	72.78
2.	10	11.43	114.36	11.44	114.36	11.44	114.36
3.	20	20.89	104.46	21.99	109.36	21.99	109.96
4.	50	51.41	102.82	53.41	106.82	53.41	106.82
5.	100	98.42	98.42	97.62	97.62	97.62	97.62
6.	200	196.89	98.45	196.89	98.45	196.90	98.45
Tianeptine							
1.	5	4.75	95.02	4.91	98.24	4.91	98.24
2.	10	8.85	88.57	8.86	88.57	8.86	88.57
3.	20	21.80	109.04	22.00	110.04	22.01	110.04
4.	50	51.31	102.62	51.31	102.62	51.31	102.62
5.	100	103.04	103.05	103.15	103.15	103.15	103.15
6.	200	194.76	97.38	194.77	97.38	194.77	97.38
Mirtazapine							
1.	5	4.83	96.64	4.93	98.64	4.93	98.64
2.	10	9.72	97.20	9.72	97.20	9.72	97.26
3.	20	20.87	104.36	20.77	103.86	20.78	103.86
4.	50	49.63	99.25	49.53	99.05	49.55	99.05
5.	100	102.24	102.24	102.44	102.44	102.43	102.44
6.	200	197.61	98.81	197.61	98.81	197.61	98.81

3.5. Interference, carryover, and matrix effect.

Matrix effects for all analytes at two distinct concentration levels (lower quality control (LQC); 5 ng/L and Higher quality control (HQC); 200 ng/L) were recorded. For both quality control levels, we observed variations of less than 20% of matrix effects for all analytes, which

is acceptable data and satisfies the validation standards. A common compound in simulated urine, saliva, and gastric fluid samples was not detected in the interference test, ensuring that the approach is selective for both ADs. Carryover was observed at the highest quantities identified from earlier injections of the target drug.

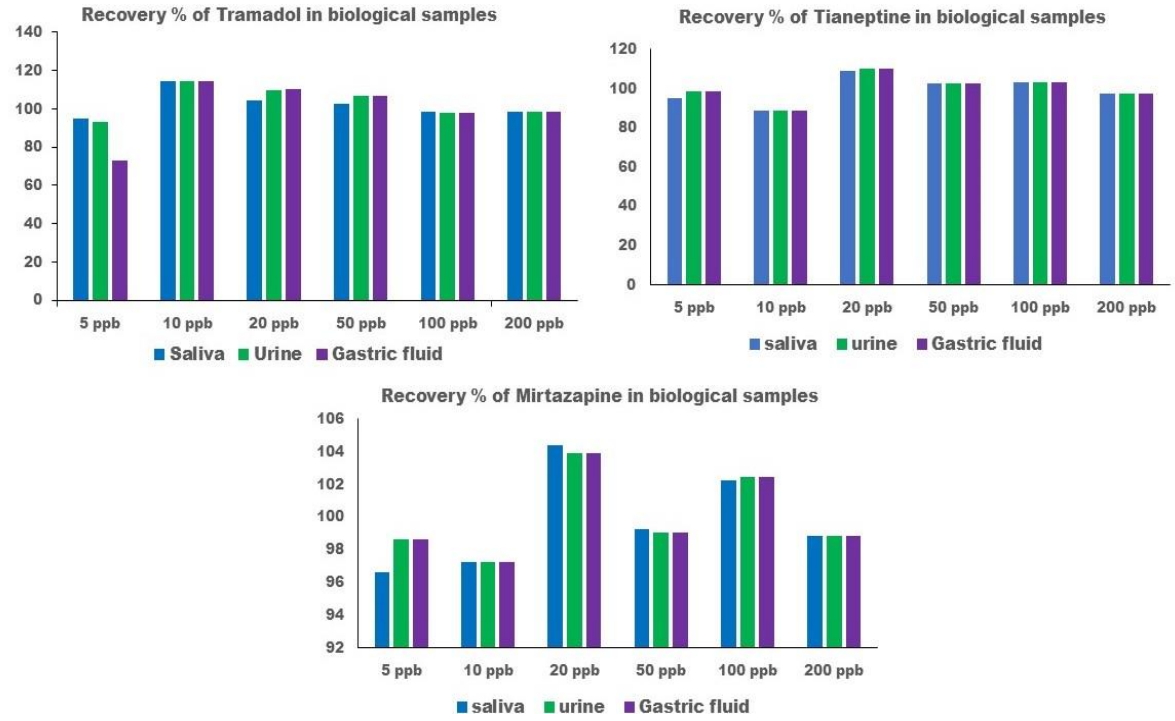

Figure 6. Bar graph of recovered TNP, TMD, and MTZ samples in biological matrices. The bars represent the recovery percentage concerning the different concentrations of drugs in samples.

The present method on LC tandem mass spectrometer could detect TMD, TNP, and MTZ in a solo run with a LOD of 5 ng/mL. Various protocols for tests on biological samples utilizing various solvents, salt combinations, and SPE sorbents were used. However, there are few research that has improved these conditions by utilizing a design of experiments and statistical data to determine the ideal conditions for TNP, TMD, and MTZ extraction[52–56]. This modified approach proves methanol is the most appropriate solvent in QuEChERS applications and is effective for a large selection of analytes. In a study conducted by Chang *et al*. (2018), methanol was used as a solvent for the extraction of heterocyclic amines, which yielded a recovery of 56% - 113 %[57]. Miao *et al*. (2015) examined the same method for detecting pesticides, using methanol as the solvent for extraction [58]. Despite the existence of studies involving only the first step of QuEChERS extraction [23], which were considered miniaturizations of the method, we decided to test and keep the clean-up step based on the original approach[42], due to the matrix complexity, especially in comparison with other biological matrices. As a result, we used the clean-up stage to eliminate any significant matrix influence, allowing us to produce clearer extracts. Furthermore, efficient cleaning prevents damage to the HPLC column, which could lead to source contamination in the long run[52]. Additionally, this method is the first to reveal such good recovery of TMD, TNP, and MTZ in ppb level from urine, saliva, and gastric fluid compared to prior findings by using LC-MS/MS[51,60–62].

Factor associated with human biological samples also plays a vital role in the result of extraction protocols. To overcome these, simulated samples come into action, giving a similar result compared to human biological matrices and overcoming factors such as high matrix

effect or recovery. As quantified ADs are easy to obtain and belong to such categories of drugs that medical experts around the world frequently prescribe, their abuse has also grown relatively with frequently reported suicide attempts.

4. Conclusions

ADs are widely prescribed drugs worldwide and getting abused in many clinical and forensic cases. The quantification of many ADs is still uncertain due to the rapid increase in ADs mortality that has been observed globally. In this work, we used a modified QuEChERS and LC-MS/MS technique to efficiently and accurately quantify three different categories of ADs in biological materials. The QuEChERS extraction was optimized using low sample and solvent consumption under the optimal conditions with methanol as the solvent, a combination of sodium acetate and magnesium sulfate salts for salting-out effect, vortex or homogenizer for agitation, and PSA with magnesium sulfate for sorbent clean-up. The entire approach was verified to meet the criteria for all analytical attributes, exemplifying specificity and accuracy for the TMD, TNP, and MTZ. The approach was validated and shown to have desirable linearity, intermediate precision, repeatability, and accuracy. The method's recovery ranged from 72-114 percent. Relative percentage standard deviation values of less than 20%, the method precision in accordance with repeatability and goodness of fit parameter R^2 was significant. As a result, the method was successfully applied to simulated biological samples, exhibiting that the designed framework is easy, efficient, and accurate in monitoring the lowest concentrations of TMD, TNP, and MTZ for life-threatening overdoses and suicidal behaviors, indicating that it could be a feasible substitute for clinical practices.

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Acknowledgments

Not Applicable

Conflicts of Interest

The authors declare no conflict of interest.

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