Validated simultaneous high-performance thin-layer chromatography—mass spectrometry method for analysis of citalopram prochlorperazine, midazolam, and chlorodiazepoxide in urine for forensic analysis

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Abstract
Drug breakthroughs and development have been a great blessing to mankind. Problem arises when these are used in non-medical context such as use for recreational purposes, using drugs outside the label directions, and use with malicious intent as found in homicidal cases. Young generations get trapped into vicious cycle of drug addiction that has deleterious effects on their mind and body. Drugs including psychomotor drugs, opioids, narcotics, inhalants, stimulants, and central nervous system depressants are easily accessible. Forensic science plays a crucial role in investigation pertaining to drug abuse and overdose. Complex biological and non-biological matrices are submitted to forensic science laboratories for analysis and interpretation of the effects of drugs/toxicants/poisons on human body and environment. The aim of this study was to develop a method for the determination of various prescription drugs in the urine using high-performance thin-layer chromatography—mass spectrometry (HPTLC—MS) technique. New combination of solvent systems was tried for the simultaneous detection of frequently used prescription drugs in urine. The method was developed and validated using HPTLC—MS for the quantitative estimation of benzodiazepines, anti-depressants, and tranquilizers in urine using a common solvent system for all the drugs. Mobile phase cyclohexane—toluene—diethylamine (7:1.5:1.5, V/V) with one drop strong ammonia solution was used as mobile phase. Method validation was carried out according to the International Council of Harmonization guidelines. The present HPTLC—MS method being simple, sensitive, precise, and accurate can be used for the detection and quantitative estimation of benzodiazepines, anti-depressants, and tranquilizers in biological specimens.

Keywords Forensic science · Drug overdose · Urine · High-performance thin-layer chromatography—mass spectrometry (HPTLC—MS) · Quantification

1 Introduction
Deaths due to abuse of drugs, especially prescription and over-the-counter (OTC) drugs, are frequently being reported across the globe and the increase in deaths due to these problems represents a challenge for physicians, public health, psychiatry, and drug-control policies. Non-medical use of such drugs has severe effects on the mental health of the person and can cause overall physiological impairment in the long run. Having a vibrant pharmaceutical industry, India is one of the largest producers of medicines in terms of quantity as well as quality [1]. Though it seems impressive, the dark side to this is that drugs are not being used for treating the health issues for which they are manufactured by the pharmaceutical companies. According to the International Narcotics Control Board (INCB) of the United Nations Office on Drugs and Crime (UNODC) states estimates, India produced 66 tons of opium in all forms, including morphine, but only 10 percent of the available morphine was directly consumed for pain management [2]. There has been a sharp rise in the demand of drugs in recent years. Health problems due to the increasing levels of pollution, use of chemically processed foods, and higher...
stress levels are not the sole reason pertaining to this substantial use of drugs. The illegitimate use of drugs by youth or any other person and the irrational prescribing of drugs are the true reasons behind this rise [3]. Although drugs are a life savior, they can be extremely fatal if taken in a dose more than what is prescribed. This illicit consumption and overdose of pharmaceutical substances are termed as drug abuse all over the globe. Substance use and dependency has become a matter of great concern at the global level. The UN World Drug Report based on the survey conducted on drug use in India in 2018 revealed a 30% increase (base year: 2009) in the population suffering from drug used is order [4]. Out of the various addictive substances used, opioids present a spike in its use followed by cannabis and synthetic drugs [4]. Apart from the addiction of these age-old drugs of abuse, what is more worrisome is the misuse of prescribed drugs due to their legitimate access from general pharma stores and mostly by online traders [5]. Irresponsible use of such drugs has several medical consequences such as dependence (physical discomfort in the absence of drug), tolerance (effect from the original dose gets shrunk leading to increased consumption), and addiction (compulsive drug seeking and use).

There are various classes of drugs that are being prescribed by a physician for the treatment of genuine health conditions but often misused by the patients. Some common ones include opioids as painkillers, CNS depressants to treat anxiety and sleep disorders, stimulants usually in the treatment for attention deficit hyperactivity disorder (ADHD), and narcolepsy [6]. There can be innumerable reasons why prescribed drugs are being misused to such a large extent. These range from their use for recreational purposes [7] to poverty [8] and then naturally psychological problems [9]. Drugs taken recreationally result in unintentional dependency as they create a feeling of euphoria inside the human body and people take large amounts just to feel high. The lack of proper knowledge and awareness about the negative consequences of irresponsible use of drugs leads to several health conditions having adverse effects on the human body in all aspects. In general, there can be any reason for the inappropriate use of prescribed drugs that may or may not lead to demise of the user, the focal point lies in the manner such misuse of drugs affects the cognitive ability of the person. It is the psychology of the person that leads him to the path of dependency or withdrawal [10]. Due to changing scenarios of employment and high expectations from society, the youth has suffered a great ordeal of psychological damage. Disorders such as insomnia, anxiety, low self-esteem, and suicidal thoughts are an all-time high. All this has led to the excessive use of sleeping pills and other psychoactive substances such as alcohol, caffeine, nicotine, and so on that make them feel relaxed and stress-free. Drug misuse occasionally leads to drug dependency, tolerance, and addiction. But it does not mean that such drugs do not have a potential to be abused. Prescribed study drugs are used by many students to keep them focused and goal-centered. General effects of misuse are loss in concentration, slowed perception, aggressiveness, and mood swings. Overdose of sedatives and tranquilizers have adverse effects on CNS and respiratory system [11] of the person. It is the psychology of the person that leads him to the path of dependency or withdrawal [10]. According to NCRB report 2019 on suicidal death rates and its causes in India, a total of 1,39,123 suicides were reported in the country during the year showing an increase of 3.4% in comparison with the previous year (2018). The statistics on the causes of suicidal deaths indicated ‘drug abuse/addiction’ (5.6%) as one of the prime reasons after ‘family problems’ and ‘illness’ (32.4% and 17.1%, respectively) [12]. Recently, Jaipur police seized intoxicants worth Rs15 cr showing a new trend of drug consumption in state [13]. The drugs were sent for forensic investigation to find out whether it was real or fake. Seized drugs were in the form of tablets and were aimed to sell to youngsters without doctor’s prescription. Police is trying to create links between the seized consignment and the whole network behind this business. With the help of forensic laboratories and intelligence agencies, it was found that all the seized drugs were manufactured in May this year. The role of forensic science cannot be overlooked in the current health crisis. The investigation of sprawling Remdesivir black marketing racket has revealed that the vials delivered to COVID (coronavirus disease) patients were fake and did not contain original drug. The examination of seized drugs done at forensic science laboratories has confirmed that spurious vials were sold in the state at exorbitant rates [14].

The increasing trend of misuse and abuse of prescription drugs in suicidal or self-inflicted and accidental cases asks for the involvement of forensic science to develop new and efficient methods using advance instruments which would be helpful in the proper analysis of drugs. This will be useful in establishing the fact whether the crime has been conducted intentionally or done under the influence of drugs or chemicals which will further make the delivery of justice easy and more effective. In the current study, the aim was to develop a method for the determination of a variety of prescription drugs, viz., benzodiazepines (midazolam, chlordiazepoxide), anti-depressants (citalopram hydrobromide and imipramine hydrochloride), and tranquillizers (prochlorperazine maleate) in the biological matrix (urine) using high-performance thin-layer chromatography–mass spectrometry (HPTLC–MS) technique. New combination of solvent systems was tried for the simultaneous detection of frequently used prescription/OTC drugs in the urine sample. The method was validated for the determination of benzodiazepines, anti-depressants, and tranquillizers in urine matrix using a common solvent system for all the drugs.
2 Experimental

2.1 Standard drugs

Standards of midazolam (MDZ), prochlorperazine maleate (PCZM), imipramine hydrochloride (IMP HCl), citalopram hydrobromide (CTP), and chlordiazepoxide (CDEP) were procured from Indian Pharmacopeia, Ministry of Health and Family Welfare, Ghaziabad, Uttar Pradesh, India.

2.2 Reagents used in mobile phase selection and preparation

Butanol (high-performance liquid chromatography [HPLC] grade), methyl ethyl ketone (HPLC grade), strong ammonia solution, ethyl acetate (HPLC grade), dimethyl formamide (HPLC grade), acetic acid (HPLC grade), ethanol (HPLC grade), propanol, toluene (HPLC grade), acetonitrile (HPLC grade), acetone (HPLC grade), methanol (HPLC grade), chloroform (HPLC grade), cyclohexane (HPLC grade), and diethylamine (HPLC grade) were supplied by Merck (Darmstadt, Germany). Pre-coated Merck silica gel 60F254 thin-layer chromatography (TLC) plates (Merck, Mumbai, India) were used for solvent system selection (10 × 10 cm) and method validation (20 × 10 cm). Other glassware, micropipette, and twin-trough development chamber (CAMAG, Muttenz, Switzerland; 20 × 10 cm) were used for the experimental work.

2.3 Preparation of standard stock solution

An aliquot of 100 µg/mL solution was prepared using MDZ, PCZM, IMP HCl, CTP, and CDEP standard dissolved in methanol. Stock solution was filtered and sonicated for 3 min and stored at 4 °C.

2.4 Selection of mobile phase and chromatographic procedure

Series of experiments were performed to find the best mobile phase for the concerned drugs. Different combinations of solvents in different proportions based on the chemistry of the drugs under study were tried. Miscibility chart for different solvents and literature review were referred for this purpose. After several attempts, optimum combination of solvents was selected as the mobile phase and the drugs giving good resolution in the chosen mobile phase were used for further study. Out of the two poorly resolved drugs: PCZM and IMP HCl, one which is in question most of the times and encountered frequently for forensic analysis was selected. With this, the final four drugs, namely, CTP, MDZ, PCZM, and CDEP (Fig. 1) were considered for accomplishing the objectives of this study.

2.5 Preparation of spiked sample

Sample of urine was collected from a healthy volunteer with no history of drug consumption for the last three months.

Fig. 1 The chemical structure of A citalopram hydrobromide, B midazolam, C prochlorperazine maleate, D chlordiazepoxide. Source Clarke’s Analysis of Drugs and Poisons, third edition, Pharmaceutical Press, 2005
The urine was screened by TLC for the presence of any drugs prior to selection for this study. Known quantity of drug was added to blank urine sample and the concentration was made to 1000 ppm. This step was repeated for each of the four drugs. Drugs being basic in nature were extracted from spiked urine sample using diethyl ether–chloroform (80:20, V/V) [15]. A small volume of liquid extraction was used for this purpose in which extraction was done in centrifuge tubes of 15 mL in such a way that the ratio of solvent to sample remained 3:1. After the addition of solvent, the contents were first vortexed at 500 rpm and then were subjected to centrifugation in centrifuge machine for 30 min. After centrifugation, the supernatant or the organic layer was pipetted out of the tubes and transferred to 1.5 mL vials for each drug.

### 2.6 Sample application

The standard solutions of the four drugs were applied on the silica gel 60F254 TLC plate in the form of bands having band width of 6 mm each with 25 µL Hamilton microsyringe (Bonaduz, Switzerland) using a CAMAG ATS 4 instrument. Linear ascending development was carried out in twin-trough development chamber. An aliquot of 10 mL of the mobile phase composed of cyclohexane–toluene–diethylamine (7:1.5:1.5, V/V) with one drop strong ammonia solution was used. The optimized development chamber saturation time was 30 min at room temperature and the length of chromatographic run was 7 cm (Fig. 2).

### 2.7 Documentation

Once the development was completed, the developed plate was dried, visualized, and scanned using CAMAG HPTLC set-up. Instrumental details for the entire set-up are given in Table 1.

### 2.8 Method validation

The HPTLC method was validated for specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and recovery, in accordance with

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**Table 1** Instrument details and chromatographic conditions for high-performance thin-layer chromatography–mass spectrometry (HPTLC–MS) analysis

| HPTLC (Instrumental details)                  | CAMAG                      |
|-----------------------------------------------|----------------------------|
| Mass spectrometer                             | LCMS 2020 (Shimadzu, Kyoto, Japan) |
| TLC–MS Interface                              | CAMAG                      |
| Autosampler                                   | CAMAG ATS 4                |
| TLC Scanner                                   | CAMAG TS 4                 |
| Software HPTLC                                 | winCATS                    |
| Software MS                                    | LabSolutions (Atlanta, GA, USA) |
| UV detection, λmax                            | 254 nm                     |

**Mass parameters**

| Mobile solvent for extracting spot            | Methanol (with 0.1% ammonium hydroxide) |
|-----------------------------------------------|-----------------------------------------|
| Flow rate of mobile solvent for extracting spot | 0.5 mL/min                              |
| Flow of nebulizing gas (nitrogen)             | 1.5 L/min                                |
| Cone voltage                                  | 15 kV                                    |
| Desolvation line temperature                  | 600 °C                                   |
| Detector voltage                              | 0.95 kV                                  |
| Mass spectrum range (scan mode)              | 50–500 m/z                               |
International Council for Harmonization (ICH) Q2 (R1) [16] guidelines.

### 2.8.1 Specificity

Specificity for the analytical method was as curtailed by comparing the standard and spiked samples. For this purpose, a blank biological matrix and standard and spiked biological matrix were run under suitable chromatographic conditions and results were obtained accordingly.

### 2.8.2 Linearity

An aliquot of 1000 µg/mL solution was prepared by transferring 375 µL of each drug from their respective stock solution into a single vial to make volume of 1.5 mL. This was used as the combined working standard solution. It was then used to obtain the linearity curve over the range of 3–7 µg/band for each substance. Different volumes (3–7 µL) of this mix were applied to TLC plate with six replicate measurement of 5 µL. Ordinary linear regression analysis was performed for comparing peak areas with their respective concentrations and results were evaluated.

### 2.8.3 Sensitivity

Sensitivity of the developed method was ascertained by evaluating LOD and LOQ as per the ICH guidelines. The calibration curve was plotted between the amount of analyte versus average response (peak area), and the regression equation with regression coefficient was obtained. Standard deviation of the responses was determined and the detection limit and quantification limit were calculated using the formula:

\[
\text{LOD} = 3.3 \times \frac{\sigma}{S} \\
\text{LOQ} = 10 \times \frac{\sigma}{S}
\]

where \(\sigma\) = standard deviation of residual regression and \(S\) = slope of calibration curve.

### 2.8.4 Precision

It determines the degree of closeness of the measured values of a same sample when applied repeatedly to multiple aliquots. As per the validation guidelines, precision can have three different meanings.

#### 2.8.5 Repeatability

Combined working standard solution (5 µL) was spotted on TLC plate six times, the plate was developed, dried, and analyzed using the same measurement procedure, the same measuring system, the same operators, and the same operating conditions at the same location. The peak area of each band and concentration for each drug were measured and relative standard deviation (RSD) for the amount of drug found was calculated.

#### 2.8.6 Intra-day precision

For this, RSD was determined by analyzing combined working standard solution of all the four drugs at application volume of 5 µL for two times on the same day.

#### 2.8.7 Inter-day precision

For this, RSD was determined by analyzing combined working solution of four drugs at application volume of 5 µL for two times on two different days.

#### 2.8.8 Intermediate precision

In this type of precision, changes are considered over a longer period of time, typically several months. This type is also known as within-lab reproducibility. In this, the results are obtained in the same lab after a gap of several months. Though all the conditions are again tried to keep constant, this type would show larger deviation in results as compared to repeatability.

#### 2.8.9 Reproducibility

This step is useful to conduct when the analytical method is standardized. In this, results are obtained by performing the similar experiment in different laboratories and, thus, also known as between-lab reproducibility.

#### 2.8.10 Accuracy and recovery

Accuracy is defined as the degree of agreement between the experimental value, obtained by replicate measurements, and the accepted reference value. It includes both trueness and precision of the results. For this validation step, spiked samples at three different concentrations, 80%, 100%, and 120%, of the target concentration were used and three individually prepared replicates at each concentration were analyzed. For each sample, theoretical value, assay value, and percent recovery were reported and mean and percent recovery for the four analytes under consideration were calculated.

### 2.9 Mass spectrum

The identification of analyte was performed using TLC–MS Interface 2, equipped with electrospray ionization (ESI)
operated in the positive ionization mode. Chromatography separation of analytes was done using the conventional methods. The TLC–MS Interface was then positioned on the band and it was operated at the semi-automatic mode. The eluted substance from the band entered into the MS source through ESI source and the m/z values of the analytes were obtained.

3 Results and discussion

The purpose of the current study is mainly focused on the development and validation of a quantitative method for the simultaneous detection of four drugs (MDZ, CDEP, PCZM, and CTP) from three different classes, namely, benzodiazepines, tranquilizers, and anti-depressants in urine samples for forensic case analysis. The technique is best suited for forensic laboratories where cases related to these drugs are frequently submitted for examination. Our findings prove that HPTLC–MS is a fast and reliable alternative/additional confirmatory technique which may be used by forensic toxicologist for apart from other techniques like HPLC and gas chromatography–mass spectrometry (GC–MS). An optimum solvent system was first developed using readily available chemicals in the laboratory. The four drugs under study were tested for good resolution in a number of solvents. The solvent system having the composition cyclohexane–toluene–diethylamine (14:3:3, V/V) with one drop strong ammonia solution provided excellent resolution among all experimented solvent systems (Fig. 3). The plates were developed in this solvent system and scanning was done using CAMAG TLC scanner. The observations were recorded in terms of retention factor (Rf) and maximum absorbance. The Rf values for MDZ, PCZM, CTP, and CDEP were 0.31, 0.79, 0.63, and 0.07, respectively. The maximum wavelength obtained was 229 nm, 257 nm, 240 nm, and 275 nm, respectively, in the same order as above. The proposed method was validated as per the ICH Q2 (R1) guidelines. Different parameters for method validation were considered and the results were recorded. The linearity was studied over the range of 3–7 µg/mL for each drug. Compounds MDZ, CTP, and CDEP showed good linearity of nominal concentration and detector response with coefficient of determination (r² > 0.99) and compound PCZM with r² = 0.98. Sensitivity was determined by estimating LOD and LOQ over the entire range of the calibration curve.

Residual standard deviation of regression was used for this determination. Using the equation of regression line for each drug as obtained from the calibration curve and MS Excel statistical tool for regression, LOD and LOQ were estimated for each drug under study. The LOD/LOQ values were found to be 0.521/1.57, 0.70/2.14, 0.53/1.61, and 0.53/1.61 µg for MDZ, PCZM, CTP, and CDEP, respectively. Other parameters of method validation that were considered involved repeatability and inter-day and intra-day precisions. RSD was estimated for all the three parameters for each drug. Repeatability for the measurement of the sample application (n = 6) was found to be <4% for each drug. Intra-day and inter-day precisions were <5% for each drug. Spiking of each drug was done in the blank urine sample and then small volume liquid extraction technique was used for the extraction of drugs using diethyl ether–chloroform (80:20, V/V). Recovery and accuracy of the method were determined by comparing the change in amount obtained from the extracted urine with respect to the standard drugs at three different levels of 80%, 100%, and 120% of the target concentration. Recovery for each drug was found to be in the range of 90–100%.

For quantification purpose, HPTLC was coupled with mass spectrometer. The spots were extracted from the HPTLC plates using CAMAG TLC–MS Interface and introduced into the mass spectrometer which produced the mass
spectrum of the individual drug. The solvent system used for MS was methanol–water (50:50, V/V). The analysis was carried out in single ion monitoring mode and positive-ion event. The positive-ion mode showed major ions [M–H]+ at m/z 325.4 for MDZ, 373.90 for PCZM, 324.30 for CTP, and 299.75 for CDEP. The method was properly developed and validated, and the results obtained were in the range of acceptance values for all parameters. Summary of all the parameters of method validation is given in Table 2 (see also Figs. 4, 5, 6).

| Compound | MDZ | PCZM | CTP | CDEP |
|----------|-----|------|-----|------|
| Retention factor (Fig. 4) (R_f) | 0.31 | 0.79 | 0.63 | 0.07 |
| Linearity range (Fig. 5) | 3–7 µg/band | 3–7 µg/band | 3–7 µg/band | 3–7 µg/band |
| Coefficient correlation ± SD | 0.99628 ± 1.73 | 0.99318 ± 2.79 | 0.99613 ± 2.54 | 0.99612 ± 1.60 |
| Repeatability (RSD%) | 3.79% | 1.04% | 1.89% | 2.27% |
| Intra-day precision | 4.78% | 3.48% | 3.09% | 2.68% |
| Inter-day precision | 4.39% | 3.37% | 3.87% | 3.02% |
| Limit of detection (µg) | 0.52 | 0.70 | 0.53 | 0.53 |
| Limit of quantitation (µg) | 1.57 | 2.14 | 1.61 | 1.61 |
| m/z Mode (Fig. 6) | 326(+) | 374(+) | 324(+) | 300(+) |
| Mean recovery % | 95.5 | 90.5 | 95.89 | 92.47 |

4 Conclusion

Drugs which are considered in this study are frequently encountered by forensic laboratories under poisoning and suicide cases. These drugs have deleterious effects on the body and thus often become the reason of death in majority of cases. It becomes imperative in the field of research to develop methods which are easy to perform, quick, and have greater accuracy in the detection of drugs and their metabolites from the biological fluids. The principle feature of the proposed method is the use of HPTLC–MS technique which provides rapid identification along with the quantification of drugs in the disputed samples. The developed confirmatory technique is simple and sensitive as it offers an easy way to analyze the drugs on a HPTLC plate by measuring optical density of separated bands. The data obtained after analysis can simply be compared with the standard curves from reference materials which are chromatographed under the same conditions. This lowers the operating costs of the analytical method and provides a high sample output. The simultaneous detection and quantification of three different classes of drugs (benzodiazepines, tranquilizers, and anti-depressants) in urine are add-on to the advantages offered by the proposed method. An optimized solvent system, using readily available chemicals, was developed which gives excellent resolution for the four drugs considered in the study. The use of single solvent system for the analysis of different classes of drugs saves time and makes this method economical. The validation of the method by the parameters defined in the ICH guidelines makes it promising and suitable for practical application.
Fig. 4 Specificity: A chlordiazepoxide, B citalopram hydrobromide, C prochlorperazine maleate, D midazolam (quantity applied: 7 µg/band)
Fig. 5  Linearity curve: A midazolam, B prochlorperazine maleate, C citalopram hydrobromide, D chlordiazepoxide
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Author contributions KLV contributed to conceptualizing, designing, supervising, and interpreting the data pertaining to this research work. PC carried out the literature review, performed the experiments, analyzed the data, and participated in drafting the manuscript. DK performed literature review, experiments, and instrumental analysis and analyzed the data. All authors discussed the results and commented on the manuscript.

Fig. 6 Mass spectrum of A chlordiazepoxide, B citalopram hydrobromide, C midazolam, D prochlorperazine maleate
Declarations

Conflict of interest There are no financial, or other, relations that could lead to a conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by the authors.

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