A novel analytical method of TFMPP and mCPP in fluids of drug addicts using LLE-GC/NPD

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Abstract. In recent years, drug-abuse problem is growing by leaps and bounds all over the world. The master minds spearheading its proliferation among the youth are difficult to identify, so drug-abuse case has become a hard nut to crack even with the help of best international experts in forensic science and criminology. Because most nations have tightened their controls on traditional drugs, the younger generation is now hooked onto new-type drugs: 1-(3- trifluoromethylphenyl) piperazine (TFMPP), 1-(3-chlorophenyl) piperazine (mCPP) and other new piperazine-drugs, acting as hallucinogens like 'ecstasy', are being consumed by vulnerable masses all over the world. However, only few research studies have focused on developing highly effective detection methods for TFMPP and mCPP in biological fluids; the number of detection methods for these new-type drugs is almost nil in China. Therefore, it is difficult to detect and prevent drug abuse cases related to piperazine drugs in China. There is an urgent need to develop some simple, fast, and reliable methods for detecting piperazine-drugs in vulnerable masses. Thus, the development of novel detection methods with high sensitivity and selectivity is a difficult task for the officials working in the department of forensic science in China. In this work, a new method was developed for the detection of piperazine derivatives: it was performed under the various specific conditions required for conducting chromatography and mass spectrometry analysis. With this novel method, TFMPP and mCPP was successfully detected with high accuracy in various biological samples. By comparing the purification effect of different solid-phase extraction columns for TFMPP and mCPP in biological fluids (urine and blood), we confirmed the validity of the novel method. In addition, this method has good linear relationship and a low detection line when GC/MS was performed for detecting TFMPP, mCPP in the biological fluids (urine and blood). It is a simple, reproducible method that is highly specific in the detection of piperazine-drugs. Thus, it is indeed a reliable method in forensic science.

Keywords: 1-[3-(Trifluoromethyl) phenyl] piperazine, 1-(3-Chlorophenyl) piperazine, TFMPP, mCPP, piperazine derivatives, GC-MS, biological fluids, forensic drug, toxicology tests

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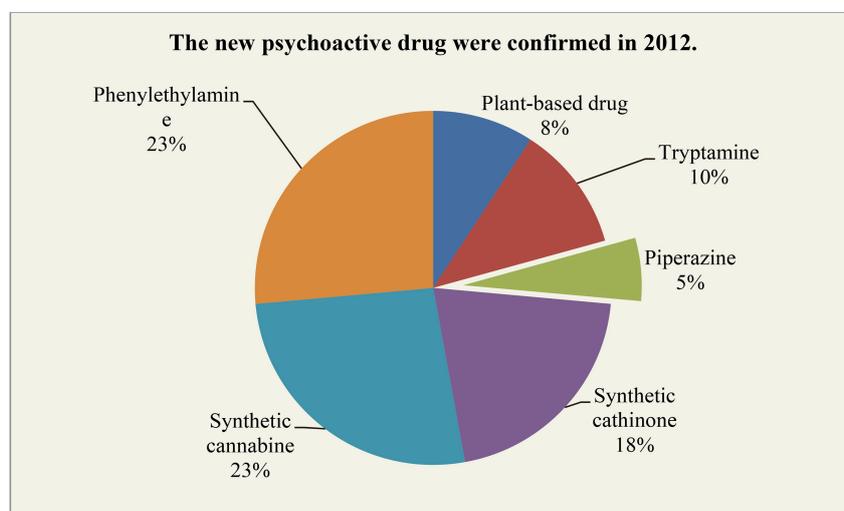
Fig. 1. The new psychoactive drug is confirmed in 2012 [24].

1. Introduction

Although the global economy has grown by leaps and bounds in the past few decades, it has also led to some serious malaise among vulnerable masses, such as youth and children. Drug abuse issues have also increased tremendously all over the world in recent times, and these menacing issues can seriously diminish the social security of any country within a very short period. People who are addicted to drugs are not just unproductive but they are also usually involved in public-order crimes. In recent years, youngsters have got hooked onto new-type drugs, such as 'party pill', 'Club drugs', etc. [1]. In new-type drugs, the most common psychoactive drug substances are as follows: synthetic cannabinoid, phenylethylamine, synthetic kathy ketone, tryptamine, and piperazine. Our aforementioned pharmacological analysis is based on the official statistics in 2012 (Fig. 1). In particular, piperazine derivatives are representative substitutes of 'ecstasy' [2]. In recent years, the incidence of piperazine drug abuse has been reported in many countries such as New Zealand, America, and Japan; however, a new uniform framework of law has not been introduced till date to prevent the rampant use of new-type drugs, so youngsters get easily hooked onto these drugs and the menace seems to be uncontrollable these days [3]. Meanwhile, many research studies today are focusing their investigation on N phenyl piperazine in order to understand the pharmacological activity of this compound [4–11]. Both TFMPP and mCPP are N-phenyl-piperazine derivatives; these compounds are drugs with mind-altering nature. Therefore, they are included in the class of illegal drugs.

To investigate and verify cases related to incessant consumption of new-type drugs and to detect addicts of new-type drugs, GC/MS is one of the most useful means of detection and so it is used extensively in the latest techniques of drug analysis. In forensic diagnosis, the blood and urine of drug addicts can be used to analyze and diagnose cases of new-type drug consumption [12]. The drug lasts for several hours in the human body; however, the half-lives of different drug abuse are also different [13–16], so it is very difficult to detect them. During biological fluid testing, the following two steps are used to determine the content of drugs: i) purification of extractand ii) analysis of testing. Presently, one of the most difficult problems in forensic science is the inability to complete the detection of drug substance rapidly, because the drug substance has a complex composition and biological fluids are the most common interfering

species during detection. The most common methods of pretreatment are protein precipitation, liquid-liquid extraction (LLE), and solid-phase extraction (SPE). On the other hand, the methods used in the detection of TFMPP and mCPP are as follows: HPLC/V, HPLC/FD, LC/MS, CZE/UV, etc. [17–23]. However, capillary zone electrophoresis has complex operations, and the related results do not have an appreciable accuracy. In this case, the results are not accurate enough to meet the standards of forensic science. This is because the sensitivity and reproducibility of UV detector is not good enough to detect TFMPP and mCPP in biological fluids. Therefore, our objective was to determine whether GC/MS was suitable for the detection of drugs in this study.

Gas chromatography-mass spectrometry (GC/MS) is the most commonly used technique in China. Moreover, GC/MS is an industry standard method used for detecting toxic substances in many biological fluids. The principle of gas chromatography is based on the distribution order of characteristic peaks of each component of a given solution; these components get separated according to their distinct adsorption capacity in the column. Thus, the analyte is successfully isolated from the sample and subjected to further investigation. Subsequently, the analyte is separated by protonation, and mass spectrometry provides information about the mass-charge ratio of the ions obtained by protonation. Thus, GC/MS exhibits high separation efficiency by effectively separating all the components of any given sample. Furthermore, owing to the large database of mass spectrometry, the integrated technique of GC/MS also ensures that the identification results are exceedingly high in most cases. All the aforementioned observations establish that GC/MS is a suitable technique for conducting qualitative and quantitative analysis of most drug poisons. With this integrated method, piperazine derivatives, such as trifluoromethylphenylpiperazine (TFMPP) and meta-chlorophenylpiperazine (mCPP), can be detected without derivatization [25]; the method's results have precision that is usually greater than 20% provided the limit of detection is 5 μg/L for a concentration linear range of 5–1000 μg/L [26].

In this paper, previous jobs were further confirmed and depended [27,28]. Gas chromatography mass spectrometry (GC/MS), a popular method of every analytical chemistry laboratory, is investigated to determine whether it can be used as an instrument analysis method for the detection of TFMPP and mCPP. Moreover, researchers have also comprehensively studied all the possible methodologies of this method, and they have suggested instrumental improvements subsequently.

2. Results

2.1. Validation of the method

2.1.1. Calibration curves
The standard working solution TFMPP-mCPP mixed solution (from "4.4.2 Sample pretreatment") was prepared and diluted with methanol to form solutions of the following concentrations: 100 μg/mL, 10 μg/mL, 5 μg/mL, 1 μg/mL, and 100 ng/mL. Furthermore, GC/MS analysis was performed according to the conditions specified in "4.4 Gas Chromatography (GC/MS) Analysis", and parallel analysis of each sample concentration was performed thrice. By plotting the TFMPP or mCPP peak areas of the standard working solutions against their concentration values, the researchers successfully obtained standard calibration curves. Table 1 presents the standard curve equations for the standard solutions of both TFMPP and mCPP, and the working curves are shown in Fig. 2.

2.1.2. Limits of quantification and detection
Table 2 presents the limits of detections (LODs) and limits of quantifications (LOQs) for the TFMPP and mCPP standard solutions. Interestingly, same values were observed for both the substances.

Table 1
Standard Curve Equations of TFMPP and mCPP standard derived by GC/MS analysis

Drug-matrix solution	Concentration range (μg/mL)	Curve equation	R^2
TFMPP	0.1–100	y = 12596x – 16930	0.9990
mCPP	0.1–100	y = 11187x + 5746.9	0.9997

Table 2
LOQs and LODs of TFMPP and mCPP by GC/MC

	LOQ (S/N $\geqslant$ 10) (μg/mL)	LOD (S/N $\geqslant$ 3) (μg/mL)
TFMPP	0.1	0.08
mCPP	0.1	0.08

Table 3
Precision study on 5 replicates

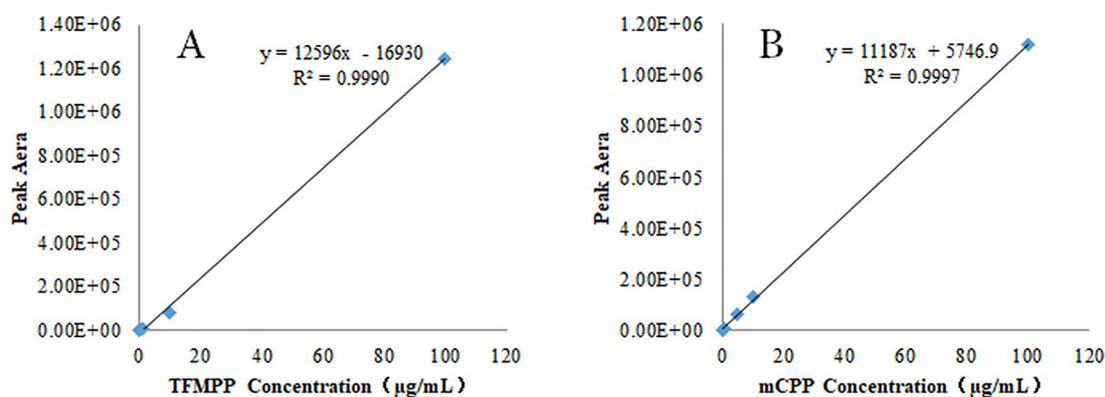|	Concentration (μg/mL)	Mean value $\pm$ %RSD	
		Intra-day	Inter-day
TFMPP	1	3.4	4.3
	10	2.5	2.7
mCPP	1	3.2	3.6
	10	2.4	3.2

Fig. 2. Calibration curve for TFMPP (A) and mCPP (B) standard working solution.

2.1.3. Precision evaluation

In this experiment, the quality control samples (QC) included only TFMPP and mCPP standard solutions with an intermediate concentration (10 μg/mL) and low concentration (1 μg/mL). The precision of this method was determined using five replicates of both TFMPP and mCPP standard solutions on the same day (intra-day precision), and the method of analysis was repeated five times every day over a period of five days (inter-day precision). As shown in Table 3, the results denote the mean values obtained after taking into account their relative standard deviation (%RSD) values. Precision studies established that the instrument used to perform this novel method has high precision, with the instrument's standard deviation being very small in magnitude.

Table 4
Standard Curve Equations and LOQs-LODs of TFMPP and mCPP in urine matrix derived by GC/MS analysis

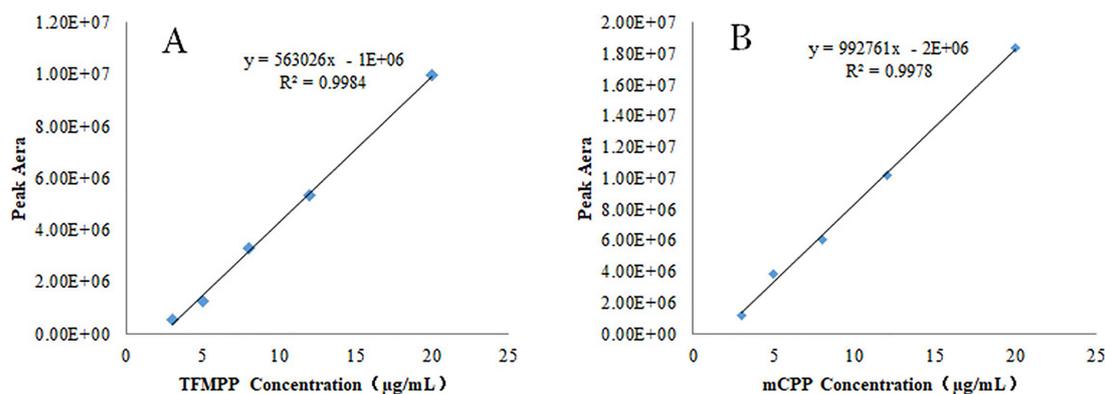| Compound	Concentration range (μg/mL)	Curve equation	R^2	LOQ (S/N $\geqslant$ 10) (μg/mL)	LOD (S/N $\geqslant$ 3) (μg/mL)
TFMPP	1–20	y = 563026x – 1E+06	0.9984	1	0.3
mCPP	1–20	y = 992761x – 2E+06	0.9978	1	0.3

Fig. 3. Calibration curve for TFMPP (A) and mCPP (B) in urine matrix.

2.2. Detection of TFMPP and mCPP in urine: Calibration and validation

2.2.1. Standard curve, limits of quantification, and detection

The standard curve was plotted after analyzing urine samples in parallel; these urine samples contained different concentrations of TFMPP and mCPP. Three replicate analyses of each urine sample were performed to determine the reproducibility of results. In this experimental analysis, 0.9 ml aliquot of five samples was taken from the blank urine. Then, mixed standard solutions of 0.1 mL were prepared using different concentrations of TFMPP-mCPP mixed solution (from "4.4.2 Sample pretreatment"); the various concentrations of TFMPP and mCPP were as follows: 1 μg/mL, 3 μg/mL, 5 μg/mL, 8 μg/mL, and 20 μg/mL in the five samples, respectively. The limits of quantitation and detection were analyzed by GC/MS according to the conditions in the method "4.4 Gas Chromatography-Mass Spectrometry (GC/MS)." While performing GC/MS method, solutions of every concentrateon were injected thrice in parallel.

The results indicate that TFMPP and mCPP have good linearity in the concentration range considered for analysis. Table 4 presents the linear equation, LOQ, and LOD. The calibration curves are shown in Fig. 3, respectively.

2.2.2. Recovery and precision studies

Two samples of urine containing 1 μg/mL and 10 μg/mL concentrations of both TFMPP and mCPP, were taken as control samples; the analysis was carried out by five times in parallel. The pretreatment method "4.5 liquid-liquid extraction method" was performed on the samples. Then, "gas-mass spectrometry (GC/MS) analysis" was performed for testing these samples. The testing procedure was performed on each sample within one day of injection, and the process of testing was conducted five times in parallel. During the testing procedure, each sample of distinct concentration was injected into the column over a period of 5 days. Then, the recovery as well as intra-day and inter-day precision were calculated

Table 5
The recovery and precision of TFMPP and mCPP by GC/MS analysis

Compound	Concentration (μg/mL)	Recovery (%)	Mean value $\pm$ %RSD%*	
			Intra-day precision (%)	Inter-day precision (%)
TFMPP	1	99.7	5.9	4.4
	10	100.5	3.6	3.7
mCPP	1	99.7	5.3	6.4
	10	98.7	7.0	6.8

Table 6
Standard Curve Equations and LOQs-LODs of TFMPP and mCPP in blood matrix derived by GC/MS analysis

Compound	Linear range (μg/mL)	Standard curve	R^2	LOQ (S/N $\geqslant$ 10) (ng/mL)	LOD (S/N $\geqslant$ 3) (ng/mL)
TFMPP	0.1–20	$y = 197516x - 6547.2$	0.9992	100	30
mCPP	0.3–20	$y = 111557x - 50991$	0.9998	300	100

for all the samples. Based on the results are presented in Table 5, we infer that the method has high recovery rate, good data reproducibility, and good instrument precision.

2.3. Detection of TFMPP and mCPP in blood: Calibration and validation

2.3.1. Standard curve, limits of quantification and detection

The standard curve was plotted after analyzing five blood samples in parallel; each sample contained a specific concentration of TFMPP-mCPP mixed solution (from "4.4.2 Sample pretreatment"), and the concentrations of all the samples were completely different. Three replicates of each sample were analyzed to ensure precision and accuracy of results. From each of the five samples of blank blood, 0.9 mL aliquot was collected. Then, 0.1 ml of mixed standard solution was taken to every samples respectively and different concentrations of TFMPP-mCPP mixed solution (from "4.4.2 Sample pretreatment") were added to each of the five aliquots. Thus, the concentrations of sample solutions were as follows: 3 μg/mL, 5 μg/mL, 10 μg/mL, 15 μg/mL, and 20 μg/mL. The limits of quantitation and detection were determined by GC/MS, and the GC/MS procedure was performed in accordance with the conditions laid down for the method "4.4 Gas Chromatography-Mass Spectrometry (GC/MS)." It should be noted that sample of each concentration was injected thrice in parallel. The results indicate that TFMPP and mCPP have good linearity in the aforementioned concentration range. Linear equation, LOQ, and LOD are presented in Table 6. The work curves are shown in Fig. 4, respectively.

2.3.2. Recovery and precision studies

Two samples of blood having 1 μg/mL and 10 μg/mL concentrations of TFMPP-mCPP mixed solution (from "4.4.2 Sample pretreatment"), were taken as control samples; the analysis was carried out five times in parallel. The pretreatment of the samples was conducted according to the procedure described in "4.5 liquid-liquid extraction method." Following successful completion of pretreatment, the samples were tested according to the procedure described in "4.4 gas-mass spectrometry (GC/MS) analysis." It should be noted that testing was completed within 1 day and injection of each sample was carried out five times. One sample of each concentration was injected over a period of five days. Then, the recovery, intra-day and inter-day precision as well as relative standard deviation (RSD%) were calculated. The results are presented in Table 7. Based on these results, we infer that the method has high recovery rate and good precision.

Table 7
The recovery and precision of TFMPP and mCPP by GC/MS analysis

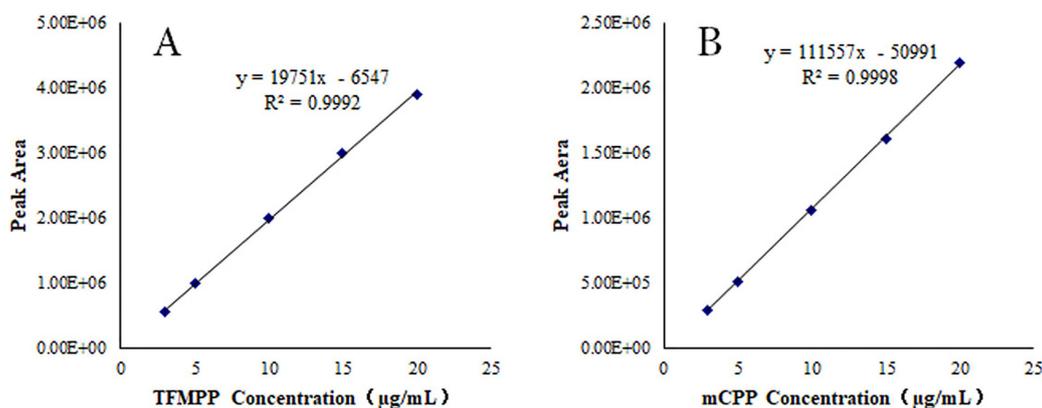|	Concentration (μg/mL)	Recovery (%)	Mean value $\pm$ %RSD%*	
			Intra-day precision (%)	Inter-day precision (%)
TFMPP	1	88.3	4.5	7.7
	10	82.8	4.4	11.6
mCPP	1	91.5	5.3	5.8
	10	89.9	7.1	5.9

Fig. 4. Calibration curve of TFMPP (A) and mCPP (B) in blood matrix.
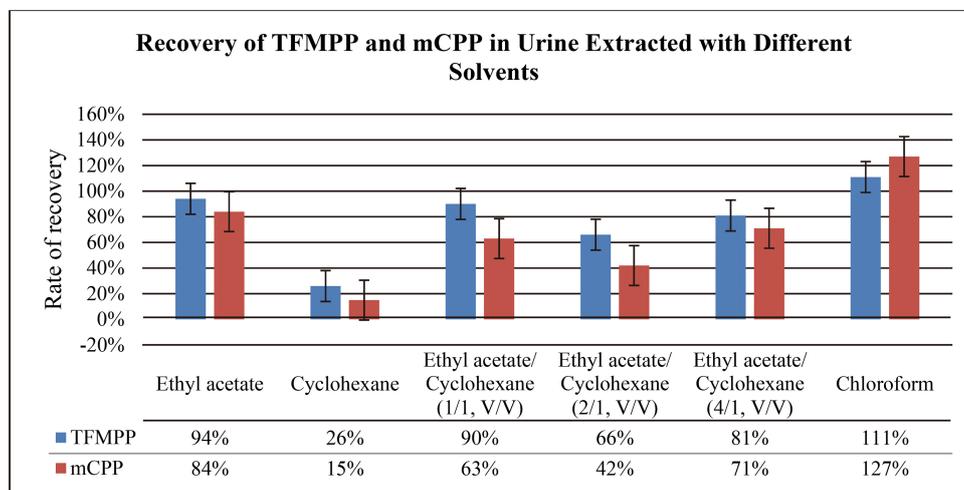
2.4. Summary

From the result, this novel method shown good linearity, low LOQ and LOD, high recovery rate and good data reproducibility for detection TFMPP and mCPP. Tested actual (urine, blood) simple also shown same result, we thank this novel method has high application value in practice.

3. Discussion

3.1. Selection of gas chromatographic columns

The first step is to select the stationary phase of chromatographic columns. Depending on the polarity of the sample, a chromatographic column is carefully selected such that the polarity of the stationary phase in the column is similar to that of the sample. With this approach, we make sure that the component to be tested (the analyte) attains its characteristic peak at the appropriate retention time, and its elution from the column is not hindered by other impurities that may otherwise act as interfering species during the elution. For example, during the analysis of non-polar samples, it is always advisable to choose only a non-polar stationary phase. Greater the similarity in the polarity of the stationary phase and the sample, greater would be the force of attraction from the fixed phase and slower would be the flow of the sample in the column. With this approach, the retention time of the sample would be surely longer. Secondly, the column length of the column must be chosen carefully: longer the chromatographic column, longer would be the sample flow time and retention time. However, an excessively long column will lead to lower column efficiency, and sample detection would be a time-consuming procedure. In addition, the column diameter and the thickness of liquid film would also affect the column efficiency.

Table 8
Comparison of recovery rates of TFMPP and mCPP in urine by different extractants

Recovery of TFMPP and mCPP in Urine Extracted with Different Solvents

	Ethyl acetate	Cyclohexane	Ethyl acetate/Cyclohexane (1/1, V/V)	Ethyl acetate/Cyclohexane (2/1, V/V)	Ethyl acetate/Cyclohexane (4/1, V/V)	Chloroform
TFMPP	94%	26%	90%	66%	81%	111%
mCPP	84%	15%	63%	42%	71%	127%

The Agilent DB-5MS is a non-polar column; its stationary phase consists of 5% phenyl-arylene and 95% dimethylpolysiloxane. The aforementioned materials exhibit a very wide range of selectivity with a minimum number of columns. In addition, the stationary phase of this column is very efficient in the retention and separation of alkaloids, anesthetics, and halogenated hydrocarbons.

In this experimental study, TFMPP and mCPP were very weak polar compounds because they had only phenyl and halogen groups in their organic structure (The polarity of a compound is determined by the nature of the functional groups present in its organic structure.). Therefore, DB-5MS column was considered to be suitable for gas chromatography. The results were as follows: column length was 30 m; inner diameter was 0.25 mm; and film thickness was 0.25 μm. Under experimental conditions, we were able to ensure that the flow rate and retention time of the mobile phase were exellent, leading to clear separation of the peaks representing TFMPP and mCPP in the samples. Thus, the sensitivity of the method was high, and the separation effect was also remarkable. In other words, the method was suitable for performing subsequent experimental analysis.
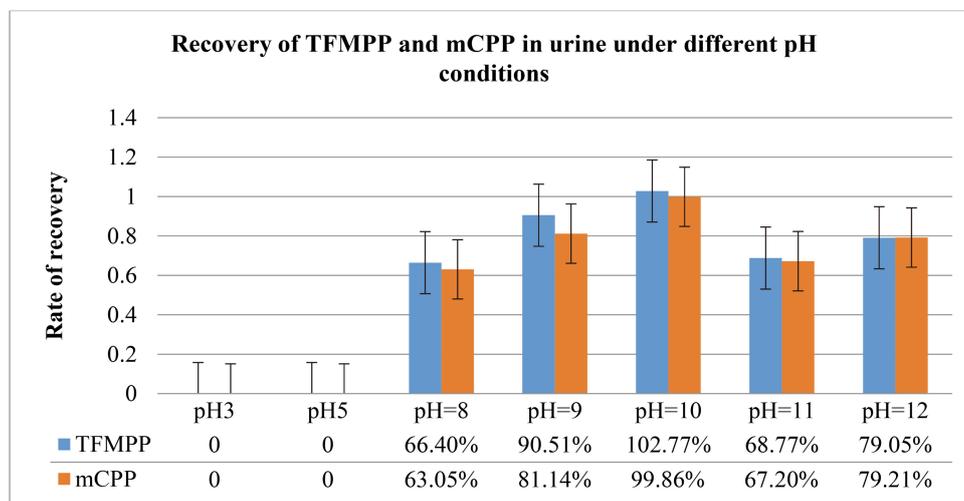
3.2. Extraction and detection of TFMPP and mCPP in urine samples

3.2.1. Optimization of liquid-liquid extraction
3.2.1.1 Effect of different solvents on the extraction rate
Both TFMPP and mCPP are soluble in many organic solvents, including acetone, ethanol, ether, benzene, cyclohexane, and chloroform. In this experiment, our aim was to investigate the effects of the following organic solvents: ethyl acetate, cyclohexane, ethyl acetate/cyclohexane (1/1, V/V), ethyl acetate/cyclohexane (2/1, V/V), ethyl acetate/cyclohexane (4/1, V/V), and chloroform. The experiment was carried out in accordance with the method "4.7 liquid-liquid extraction experiment", and the results were presented in Table 8. Based on the results, it can be concluded that the extraction rate of cyclohexane is low, so its recovery from the solvent medium is low. In contrast, the extraction recovery rate of chloroform is high; however, its main drawback is the significant interference caused by impurities. The extraction recovery rate of ethyl acetate is high and the resolution of its peaks is clearly observed in the chromatogram; the characteristic peaks of ethyl acetate are also having a well-defined shape.

Table 9
A comparison of the recovery rates of TFMPP and mCPP in urine under different pH conditions

Recovery of TFMPP and mCPP in urine under different pH conditions

	pH3	pH5	pH=8	pH=9	pH=10	pH=11	pH=12
■ TFMPP	0	0	66.40%	90.51%	102.77%	68.77%	79.05%
■ mCPP	0	0	63.05%	81.14%	99.86%	67.20%	79.21%

In the blank urine chromatogram obtained with ethyl acetate, the impurities are very few in number. Therefore, ethyl acetate was selected as the extraction agent instead of other agents; the remaining agents, such as benzene and ether solvent, had more toxicity and poor parallelism.

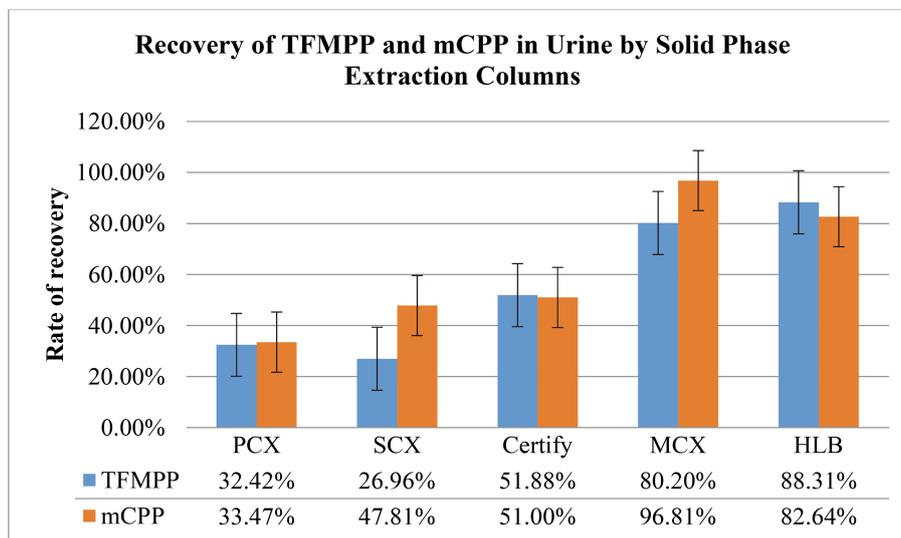### 3.2.1.2 Effect of solution pH on the extraction rates

TFMPP and mCPP are both weakly basic in nature, property pH is very important for extraction efficiencies, so the extraction efficiencies were investigated at different pH values in this experiment, the experiment procedure was carried out in accordance with the method "4.7 liquid-liquid extraction experiment". As shown in Table 9, TFMPP and mCPP at acid condition (pH = 3 and 5) cannot be detected, it maybe due to acid-base neutralization reaction make TFMPP and mCPP form a salt. However, under alkaline conditions the recovery showing excellent results, when pH = 10, the recovery reach optimum. So, pH = 10 was selected as the extraction pH.

3.2.2. Effects of solid phase extraction columns on the purification of TFMPP and mCPP

In this experiment, solid phase extraction columns, such as Oasis® SCX, Oasis® MCX, Oasis® PCX, Oasis® HLB, and BondElute, were used extensively. The purification effects of these aforementioned columns were examined in this study. In the columns Oasis® SCX and Oasis® PCX, the solid phase contained quaternary ammonium as functional groups; these chemical species are preferred as they are weak anionic compounds; the column Oasis MCX contained sulfonic strong cation exchange groups, and this chemical species has better extraction effects on alkaline compounds; Oasis® HLB Adsorbent is a reverse adsorbent with good water infiltration, so it is commonly used as a general-purpose adsorbent for acidic, neutral, and basic compounds; Bond Elut Certify is an ion-exchange and nonpolar solid phase containing mixed mode adsorbent, which is mostly used for the alkaline extraction of components from urine, plasma, serum, blood, and other biological matrix (cationic drugs).

In the aforementioned solid phase extraction columns, the extraction process was carried out according to the method "4.8 Solid Phase Extraction". The procedure performed while testing samples in Oasis® HLB is as follows: the sample was pretreated according to the method "4.6 Sample Pretreatment." Then, 1 ml of the pretreated sample was diluted with 4 mL of deionized water and mixed thoroughly for 10 min.

Table 10
A comparison of recovery rates of TFMPP and mCPP in purified urine by different solid phase extraction columns

Recovery of TFMPP and mCPP in Urine by Solid Phase Extraction Columns

	PCX	SCX	Certify	MCX	HLB
■ TFMPP	32.42%	26.96%	51.88%	80.20%	88.31%
■ mCPP	33.47%	47.81%	51.00%	96.81%	82.64%

Then, the resultant suspension was centrifuged at the rate of 8000 r/min for 15 min. Thereafter, HLB column was activated using 1 mL methanol and 1 mL ionized water, respectively. The spare sample was passed through the column; the column was subsequently rinsed with 1 mL of water and 1 mL 5% methanol water, and the eluent was discarded. The solid phase extraction column was centrifuged to remove the remaining eluent. Finally, the column was eluted with 5 mL of methanol as eluent. The eluate was collected and dried at room temperature using a nitrogen blowing instrument. Then, it was transferred to a sample vial and dilute with methanol to 100 mL for the purpose of testing.

The results are presented in Table 10. The results indicate that in each solid phase extraction column, the eluent is always colorless and transparent. The extraction of TFMPP and mCPP in urine can be performed with maximum efficiency in Oasis® MCX column. In this case, the results obtained are the best in terms of peak shape, recovery, and parallelism.

3.3. Extraction and detection of TFMPP and mCPP in blood

3.3.1. Optimization of liquid-liquid extraction
3.3.1.1 Effect of different solvents on the extraction rate

Both TFMPP and mCPP are soluble in acetone, ethanol, ether, benzene, cyclohexane, chloroform, and other organic solvents. To obtain better extraction results, we determined the effects of extraction with different solvents, such as the ethyl acetate, cyclohexane, ethyl acetate/cyclohexane (2/1, V/V), ethyl ether, benzene, chloroform and chloroform/isopropanol (4/1, V/V). This is because proteins and fats existed as impurities in blood samples. The experiment was carried out according to the method "4.7 Liquid-Liquid Extraction Experiment". The results are presented in Table 11. It was observed that the extraction of the supernatant was yellow in color, because it contained a certain fat in significant amount. In addition, the chromatographic peaks also showed serious interference from impurities. The mixed solvent of chloroform/isopropanol (4/1, V/V) exhibits both the advantages of chloroform, that is, r alkaloids and isobutanol show high solubility in this solvent when it is used for protein precipitation.

Table 11
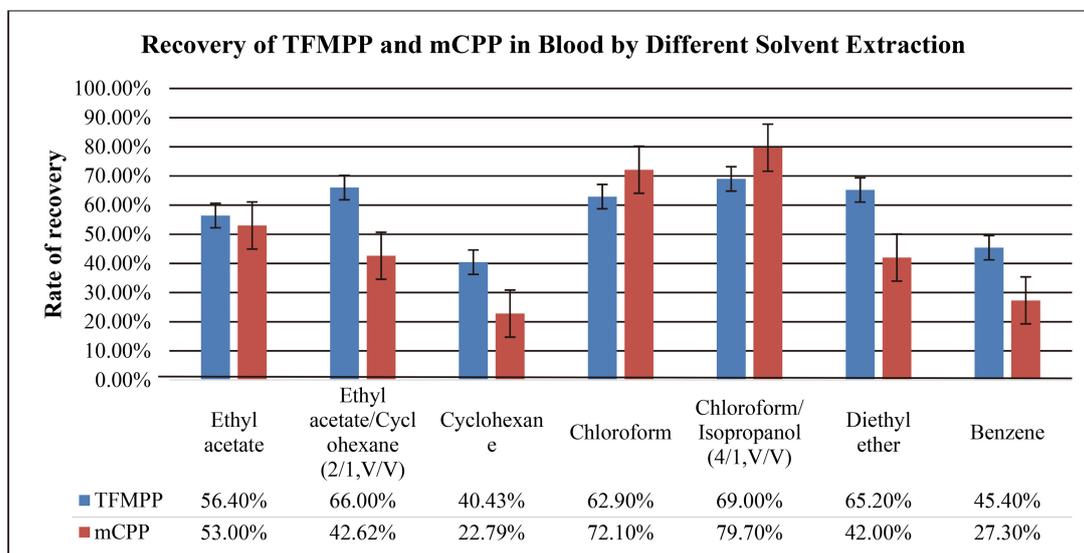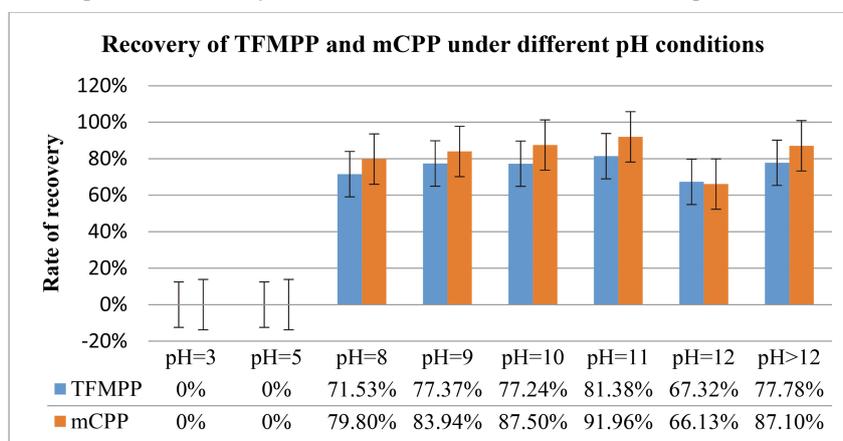Comparison of recovery rates of TFMPP and mCPP in blood by different solvent extraction

Recovery of TFMPP and mCPP in Blood by Different Solvent Extraction

	Ethyl acetate	Ethyl acetate/Cyclohexane (2/1,V/V)	Cyclohexane	Chloroform	Chloroform/ Isopropanol (4/1,V/V)	Diethyl ether	Benzene
■ TFMPP	56.40%	66.00%	40.43%	62.90%	69.00%	65.20%	45.40%
■ mCPP	53.00%	42.62%	22.79%	72.10%	79.70%	42.00%	27.30%

Table 12
Comparison of recovery rates of TFMPP and mCPP under different pH conditions

Recovery of TFMPP and mCPP under different pH conditions

	pH=3	pH=5	pH=8	pH=9	pH=10	pH=11	pH=12	pH>12
■ TFMPP	0%	0%	71.53%	77.37%	77.24%	81.38%	67.32%	77.78%
■ mCPP	0%	0%	79.80%	83.94%	87.50%	91.96%	66.13%	87.10%

The recovery rate is the highest and the shape of the peak is also good. Therefore, the mixed solvent chloroform/Propanol (4/1, V/V) was selected as the extractant.

3.3.1.2 Effect of solution pH on the extraction rates

Both TFMPP and mCPP are weakly alkaline in nature. The extraction efficiencies were investigated at different pH in this work. The experiment was carried out in accordance with the method "4.7 liquid-liquid extraction experiment", and the results are presented in Table 12. The results indicate that the two substances cannot be detected at pH 3.0 and 5.0. The best recovery rate was achieved at pH 11.0, so pH 11.0 was selected as the pH for the extraction process.

Table 13
Comparison of recovery rates of TFMPP and mCPP in blood by solid phase extraction columns

Recovery of TFMPP and mCPP in blood by solid phase extraction columns

	PCX	SCX	Certify	MCX	HLB
■ TFMPP	8.00%	24.00%	29.85%	30.46%	71.08%
■ mCPP	9.43%	18.49%	19.25%	43.77%	77.36%
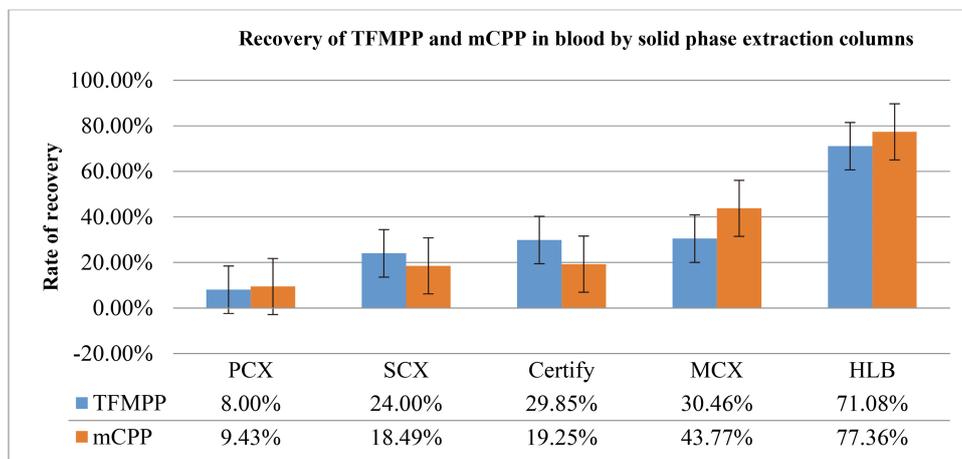
3.3.2. Effects of solid phase extraction columns on the purification of TFMPP and mCPP

The purification effects of solid phase extraction columns, such as Oasis® SCX, Oasis® MCX, Oasis® PCX, Oasis® HLB, and Bond Elute, have been examined in this study. In all the solid phase extraction columns, the same procedure was used for testing samples: the sample was pretreated according to the method "4.6 Sample Pretreatment." Then, 1 ml of the pretreated sample was added to 4 mL of phosphate buffer, and the pH was adjusted to 6. The sample was mixed with the buffer solution for 10 min, and then it was centrifuged at the rate of 8000 r/min for 15 min. The solid phase extraction columns were activated using 1 mL of methanol, deionized water, and phosphate buffers (pH = 6.0), respectively. The spare sample was passed through the column, which was then rinsed with 3 mL of water and methanol. Finally, the eluent was discarded from the column. The solid phase extraction columns were centrifuged to remove the remaining eluent. Finally, each column was eluted using 5 ml of 5% ammoniated methanol as the eluent. Each eluate was collected and dried at room temperature using a nitrogen blowing instrument. Then, the dried eluate was transferred to a sample vial and dilute with methanol to 100 mL for the purpose of testing.

According to the results presented in Table 13, PCX eluent was brown in color and it had a low recovery rate; Bond Elute certify and SCX eluents were clean but they also had low recovery rates; MCX eluent had a yellowish color, but it also had a low recovery rate and heavy tailed peak; HLB eluent was light brown in color and had the best chromatographic characteristics: the shapes of the characteristics peaks were good, the parallel results were nice, and the recovery rate was the highest among all the separated species. The results indicate that the purification effect brought about TFMPP and mCPP on the blood samples was excellent provided Oasis® HLB column was used for extraction.

4. Instruments and methods

4.1. Experimental set up

The following instruments were used in the experiment: Shimadzu GC 2010 PLUS Gas Chromatography (Shimadzu, Kyoto, Japan); Thermo Scientific Biofuge Primo R High Speed Centrifuge (SORVALL,

Inc., USA); EYELA high-speed oscillator (Tokyo physical and chemical equipment Co., Ltd, Japan); Millpore Simplicity Pure Water Preparation System (MILLIPORE, Corporation, France); Cole-Parmer Ultrasonic Oscillator (Cole-Parmer Instruments, Corporation USA).

4.2. Materials

The following reagents were used in the experiment:1-(3-trifluoromethylphenyl) piperazine (TFMPP) (purity 99.0%), 1-(3-chlorobenzene) piperazine (mCPP) (purity 99.0%), highly pure water, methanol, ethyl acetate, trichloromethane (AR), acetone (AR), ether (AR), isopropyl alcohol (AR), cyclohexane (AR), and benzene (AR). Furthermore, the following solid phases were used in chromatographic process of separation: Oasis® SCX, Oasis® MCX, Oasis® PCX, Oasis® HLB (3 mL), BondElute certify (3 mL).

To detect the traces of TFMPP and mCPP in biological samples, various standard stock solutions and working standards were prepared in the experiment. The following biological samples were tested in the experiment: blank urine samples (collected from healthy, non-smoking history volunteers) and blank whole blood samples (collected from Beijing Fuxing Hospital).

4.3. Preparation of standard stock solution and standard working solution

In the experiment, 1.00 mg/mL TFMPP and 1.00 mg/mL mCPP stock solutions were carefully diluted with methanol to form standard working solution at following concentrations: 100 μg/mL, 10 μg/mL, 1 μg/mL, 100 ng/mL, and 10 ng/mL. Using 1.00 mg/mL TFMPP and 1.00 mg/mL mCPP stock solutions, we prepared a mixed standard working solution (**M**) of of 10 μg/mL concentration. The resultant solution was sealed and refrigerated.

4.4. Gas chromatography-mass spectrometry (GC/MS) measurements

4.4.1. Calibration

The following conditions were applied while performing GC/MS measurements: GC-2010-PLUS/MS column, Solution Release 2010 workstation (Shimadzu, Kyoto, Japan) was used to analyze samples:

Chromatographic column: DB-5MS (30 m × 0.25 mm × 0.25 μm); Temperature programming: start the column at 80°C and maintain the temperature for 2 min. Then, increase the column temperature at a steady rate of 30°C/min till it attains a high temperature of 280°C. Then, maintain the column at 280°C temperature for 17 min; Carrier gas and tail blowing: high purity nitrogen, flow rate at 15 mL/min; Transmission line temperature: 250°C; Injection method: split injection, split ratio of 10:1; Mass spectrometry conditions: Electron bombardment ionization source (EI), Ionization temperature of 70 eV, interface temperature of 280°C, ion source temperature of 200°C; automatic tuning mode, the multiplier voltage 1102 V, emission current 100 μA; Solvent delay time: 2.5 min; acquisition mode: full scan m/z 40–450.

As shown in Fig. 5, the total ion current chromatogram of TFMPP and mCPP was analyzed by the chromatographic and mass spectrometric conditions mentioned in the above paragraph. The TFMPP and mCPP mass spectra are shown in Fig. 6. For the chromatographic peak that appeared at 6.537 min, the characteristic ion fragments m/z188, m/z230, m/z172 were in complete agreement with the characteristic peaks of TFMPP mass fragment obtained from the NIST library. For the chromatographic peak that appeared at 7.484 min, the characteristic ion fragment m/z154, m/z196, m/z56 were in complete agreement with the characteristic peaks of mCPP mass fragment obtained from the NIST library.
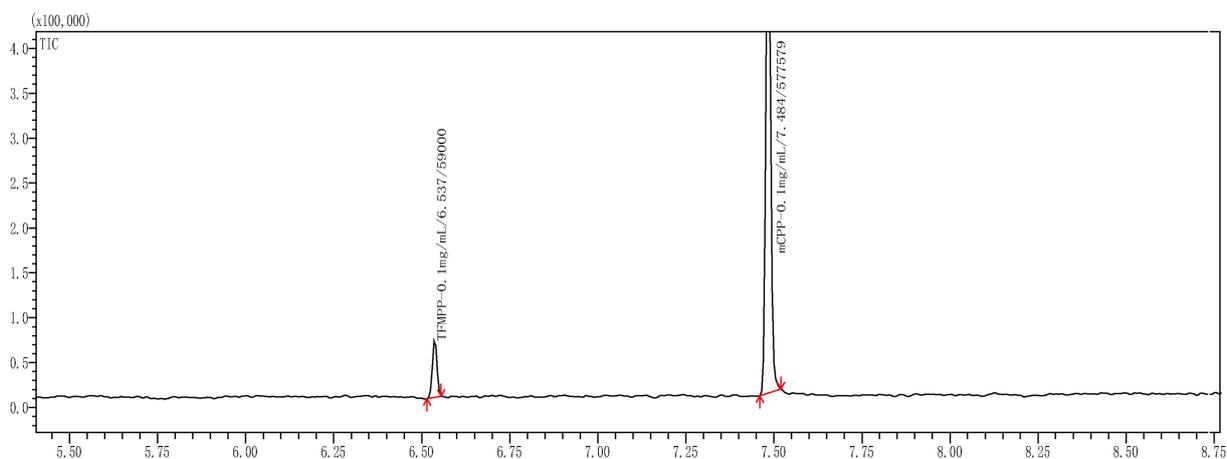
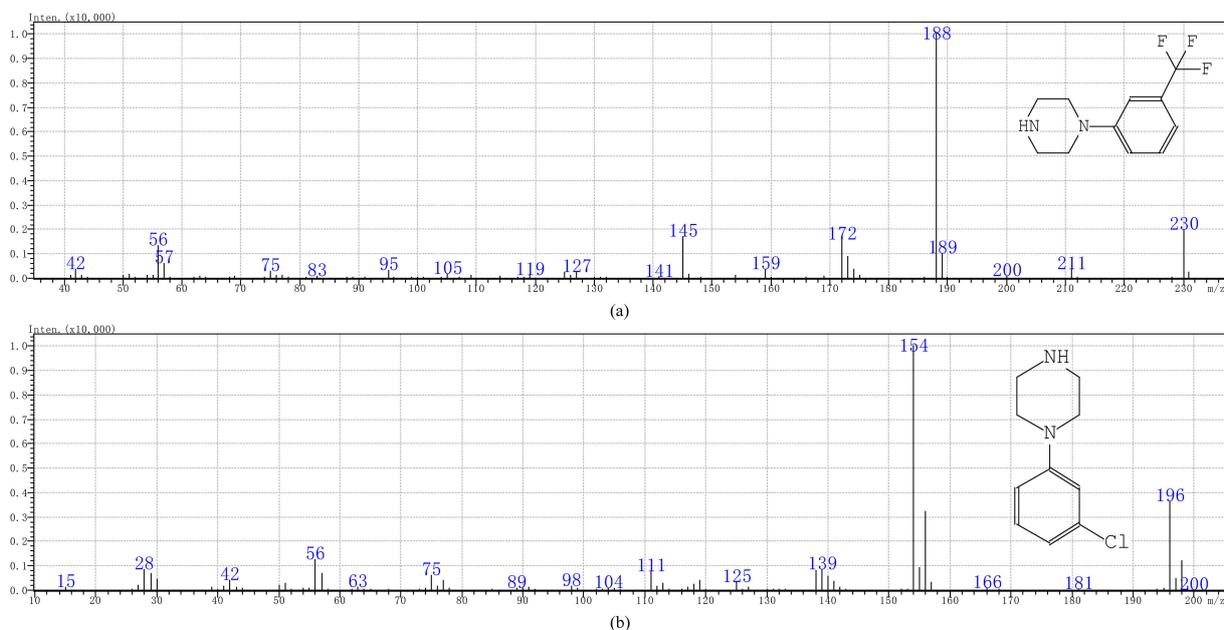Fig. 5. Gas chromatogram of TFMPP and mCPP.

Fig. 6. Mass spectra of TFMPP(a) and mCPP(b) standards.

4.4.2. Sample pretreatment

In this experiment, 0.1 mL of TFMPP-mCPP mixed solution of 10 μg/mL concentration was added to 0.9 mL blank urine and shaken thoroughly to obtain 1 μg/mL of a mixed solution containing urine and TFMPP-mCPP. The mixture was stored statically for two hours; then, it was preserved for further use.

4.5. Liquid-liquid extraction methods

4.5.1. Extraction and detection of TFMPP and mCPP in urine

The sample was subjected to pretreatment according to the method "4.4.2 Sample Pretreatment." Then, the pH of 1 ml of the pretreated samples was adjusted to pH = 10.0. Thereafter, 3 mL ethyl acetate was
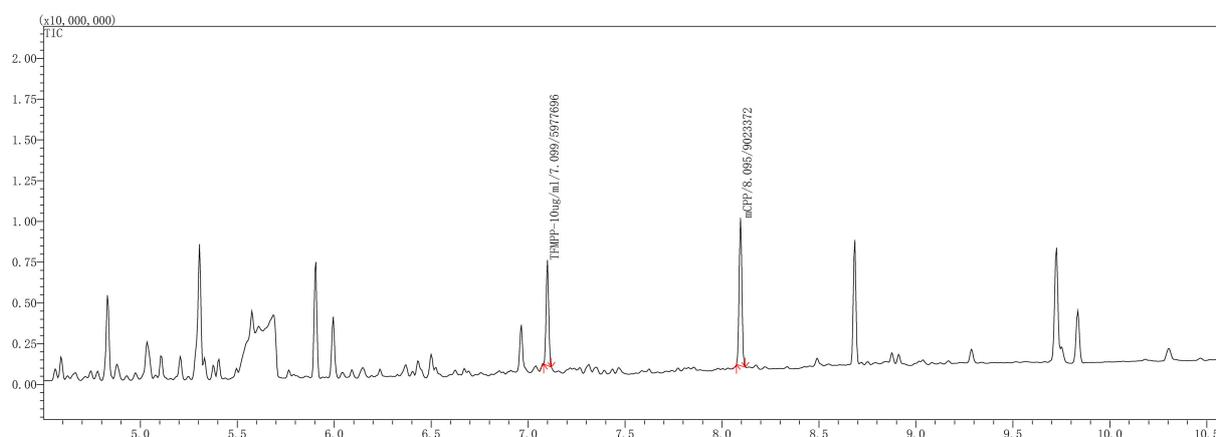
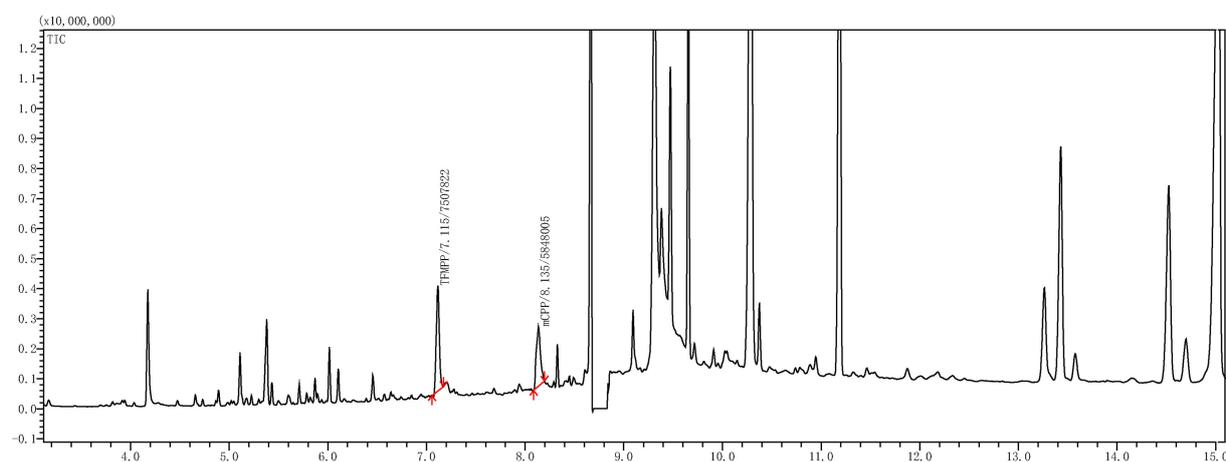Fig. 7. Liquid-liquid gas chromatogram of the urine-TFMPP/mCPP solution.

Fig. 8. Liquid-liquid gas chromatogram of the blood-TFMPP/mCPP solution.

added for the purpose of extraction, and the resultant suspension was vortexed for 10 min. Finally, the suspension was centrifuged at the rate of 8000 r/min for 15 min. The supernatant was collected and dried by nitrogen blowing instrument at room temperature. Then, the dried supernatant was transferred to a sample vial containing a constant volume of 100 μL methanol for the purpose of testing; the test was repeated five times in parallel. The gas chromatogram of TFMPP-mCPP solution containing urine was obtained after GC/MS detection, as shown in Fig. 7.

4.5.2. Extraction and detection of TFMPP and mCPP in blood

The sample was subjected to pretreatment according to the method "4.4.2 Sample Pretreatment." Then, the pH of 1 mL of the pretreated sample was adjusted till its pH = 11.0. Thereafter, 3 mL chloroform/isopropyl alcohol (4/1, V/V) was added for extraction and vortexed for 10 min. Finally, the suspension was centrifuged at a rate of 8000 r/min for 15 min. The supernatant was collected and dried by nitrogen blowing instrument at room temperature. Then, it was transferred to the sample vial containing a constant volume of 100 μL methanol for the purpose of testing; the test was performed five times in
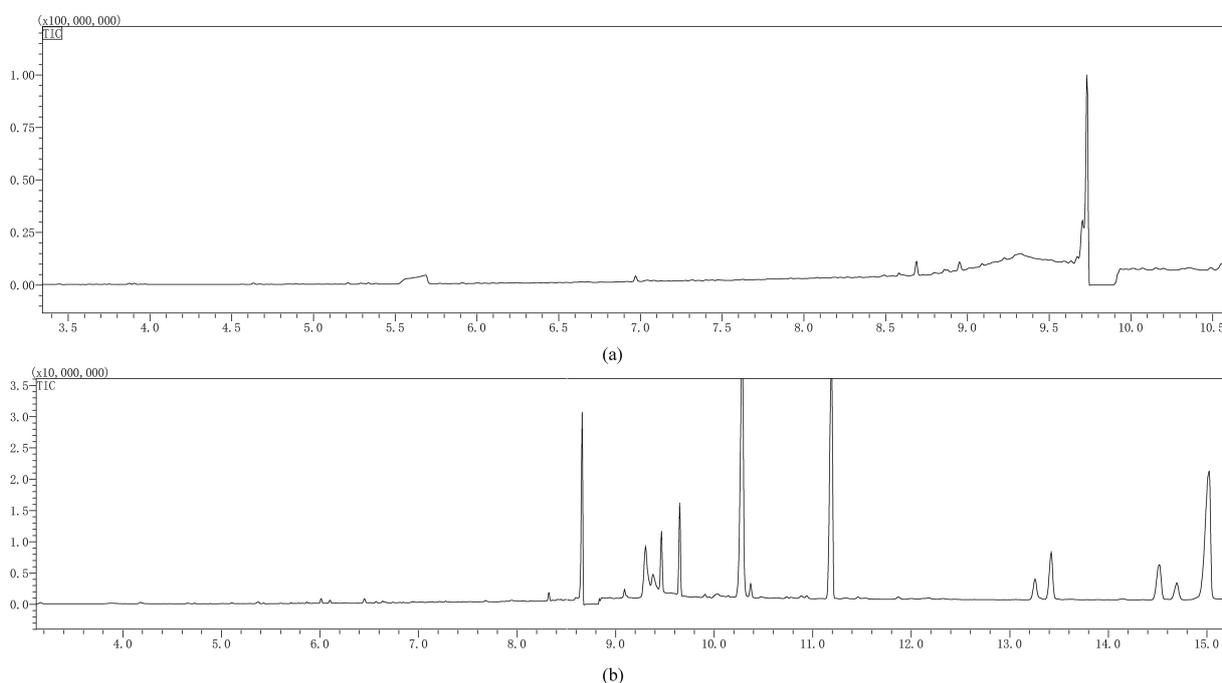
Fig. 9. Gas chromatogram of blank urine (a) and blank blood (b).

parallel. The gas chromatogram of TFMPP-mCPP solution containing added blood was obtained after GC/MS detection, as shown in Fig. 8.

4.6. Solid phase extraction

In this extraction, the sample was pretreated according to the method "4.4.2 Sample Pretreatment." Then, 1 ml of the pretreated sample was added to 4 mL phosphate buffer and its pH was adjusted to 6. Then, it was vortexed for 10 min, and finally centrifuged at a rate of 8000 r/min for 15 min. The solid phase extraction column Oasis® MCX was activated with 1 mL of methanol, deionized water and pH 6.0 phosphate buffers, respectively. Then, the spare sample was passed through the column, and 3 mL of water and methanol was used to rinse the column. Finally, the eluent was discarded from the column. The solid phase extraction column was centrifuged to remove the traces of the eluent remaining in the column. Finally, the column was eluted using 5 ml of 5% ammoniated methanol as the eluent. Then, the eluate was collected and dried at room temperature using a nitrogen blowing instrument. Finally, it was transferred to a sample vial and tested with a constant volume of 100 μL methanol.

4.7. Specificity experiment

In this experiment, 1 mL of each type of sample solution (blank urine, blood, and standard mixture of TFMPP-mCPP) has been analyzed according to the methods described in "4.5 liquid-liquid extraction method" and "4.4 gas chromatography and mass spectrometry (GC/MS) analysis". With these methods, we easily obtained the chromatograms of the standard mixture of TFMPP-mCPP (Fig. 8), the blank urine (Fig. 9a), and the f blank blood (Fig. 9b). The results indicate that the impurities in blank urine or blank blood do not interfere with the GC/MS analysis of TFMPP-mCPP solution. The method established in this study has good specificity.

5. Conclusion

This paper is on the research of the prior treatment methods (liquid-liquid extraction, solid-phase extraction) and GC/MS detection for TFMPP and mCPP in biological fluids (urine and blood) at the blankness of the test and regulation at present. This method possesses the practical value for its advantages of simple operation, high recovery, high sensitivity, and low detection limit, while the instruments are already available in lab. It is simple, reproducible and specific for detecting the piperazine-drugs provides a reliable method in forensic science.

Acknowledgments

Conflict of interest

None to report.

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