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Short communication

Extraction and determination of some psychotropic drugs in urine samples using dispersive liquid–liquid microextraction followed by high-performance liquid chromatography

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ABSTRACT

A simple, rapid and sensitive method termed as dispersive liquid–liquid microextraction (DLLME) combined with high-performance liquid chromatography-ultraviolet detector (HPLC-UV) has been proposed for the determination of three psychotropic drugs (amitryptiline, clomipramine and thioridazine) in urine samples. The determination was performed on a C_8 column under the optimal chromatographic conditions (mobile phase: ammonium acetate (0.03 mol L^{-1}, pH 5.5)–acetonitrile (60:40, v/v); flow rate: 1.0 mL min^{-1}; detection wavelength: 238 nm). Several factors influencing the extraction efficiency of the target drugs, such as pH, extraction and disperser solvent type and their volume, extraction time and ion strength were studied and optimized. Under the optimal DLLME conditions, the absolute recoveries of amitryptiline, clomipramine and thioridazine from the urine samples were 96, 97 and 101%, respectively. The detection limits (LODs) and quantification (LOQs) of the proposed approach were 3 and 10 ng mL^{-1} for amitryptiline, 7 and 21 ng mL^{-1} for clomipramine, and 8 and 25 ng mL^{-1} for thioridazine, respectively. The relative standard deviations (RSDs) for nine replicate determinations at 0.100 μg mL^{-1} level of target drugs were less than 4.8%. Good linear behaviors over the investigated concentration ranges were obtained with the values of $R^2 > 0.998$ for the target drugs. The proposed method was successfully applied to the real urine samples from two female patients under amitryptiline and clomipramine treatment, respectively.

1. Introduction

Psychotherapeutic drugs are used for treatment of psychiatric disorders in clinical therapy. Those include antipsychotics, antidepressants, mood stabilizers, anxiolytics, psychostimulants, and nootropics [1]. Among them, amitryptiline and clomipramine, which were the most prescribed tricyclic antidepressants (TCAs) early between 1960 and 1980, have been especially for endogenous major depression treatment by blocking the postsynaptic receptors and inhibiting the reuptake of different neurotransmitters [1]. In addition, thioridazine, as a phenothiazine, is also intensely prescribed for treatment of schizophrenia on the base of the blockade of nervous impulses from the central nervous system by interdiction of dopamine receptors. However, on the basis of clinical experience, those antidepressants in overdose would cause severe side-effects such as myocardial depression and ventricular arrhythmia and sometimes cause patients death [2]. Similarly, overdoses of thioridazine would cause some adverse risks such as coma, miosis, and respiratory depression [3]. Despite the disadvantages described above, those psychotherapeutic drugs are still prevailed in the outpatient clinic especially for many senior patients due to their cheapness. Therefore, the monitoring of those drugs in clinical study is significant, and the establishment of methodologies for those drugs in biological matrices is essential for patients' safety [4–7].

The general methods for analyzing psychotropic drugs in different biological samples are based on combining a very efficient separation technique with a sensitive detection technique. At present, numerous separation techniques, including high-performance liquid chromatography (HPLC) [4,6,7], gas chromatography (GC) [8] and capillary electrophoresis (CE) [3,9], have been employed for the analysis of psychotropic drugs. Among those methods, HPLC has been considered as the most efficient and robust specific techniques due to some merits of convenience, simple operation, strong separation ability and wide sample application. Various detection techniques have been applied to accurately determine the concentration of psychotropic drugs in various samples, those include UV spectrophotometry [3,4], fluorimetry [5], flame ionization detection (FID) [8], electrochemical detection [10], chemiluminescence method [11] and mass spectrometry [12]. Of all those detection methods, UV is widely used to couple with HPLC or CE due to its low cost, acceptable sensitivity and easy for on-line determination. However, in many cases, owing to matrix interfer-

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ence and insufficient instrumental detection limit for (ultra)trace psychotropic drugs in real biological samples, direct chromatographic separation and determination of those species is difficult [13]. Therefore, in order to obtain accurate, reliable and sensitive results, a separation/preconcentration method is required prior to chromatographic separation of psychotropic drugs [13].

Some separation/preconcentration procedures including liquid–liquid extraction (LLE) [14] and solid-phase extraction (SPE) [4,6] have been applied for the determination of psychotropic drugs. However, LLE is tedious, time-consuming and normally requires large amounts of organic solvents that are potentially hazardous to human health. While SPE requires a specific device loaded with certain adsorption material as well as a high-pressure delivery system that can be relatively expensive. It should be noted that some of microextraction methods such as hollow fiber-based liquid phase extraction [7] and fiber-in-tube solid-phase microextraction [8] have been successfully used for the analysis of some drugs in the biological fluids. Recently, a novel liquid phase microextraction techniques named as dispersive liquid–liquid microextraction (DLLME) has been proposed by Assadi and co-workers [15], which is based on a ternary component solvents extraction system including extraction solvent, disperser solvent and aqueous samples containing analyte of interest. DLLME is attracting more and more people's attention due to its superior advantages of high enrichment factor, high recovery, low cost, rapid and easy operation [15]. Up to now, the method has been widely used for the analysis of organic compounds in environmental water samples, including polycyclic aromatic hydrocarbons (PAHs) [15], organophosphorus pesticides (OPPs) [16], organosulfur pesticides (OSPs) [17], chlorophenols (CPs) [18], phthalate esters [19], polybrominated diphenyl ethers [20], etc. In addition, its application has been extended in the field of trace elemental analysis in environmental water samples [21,22]. Among those methods, DLLME is widely applied in the preparation of environmental water samples and rarely applied for the analysis of drugs in complex biological fluids.

In the present study, DLLME was applied for the extraction and preconcentration of three psychotropic drugs (amitryptiline, clomipramine and thioridazine) in urine samples prior to their determination by HPLC-UV. The factors influencing the extraction efficiency and determination were evaluated in detail. The developed method was successfully applied to real urine sample analysis.

2. Experimental

2.1. Chemicals and reagents

Amitryptiline, clomipramine and thioridazine were kindly supplied by Hunan Dongting Pharmaceutical Co. Ltd. (Hunan, China) and their contents were all above 99%. Methanol, acetonitrile and dichloromethane were purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Chloroform and carbon tetrachloride were purchased from Tianjin No.3 Chemical Reagent Factory (Tianjin, China). Chlorobenzene, acetone and isopropanol were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Among the above-mentioned reagents, methanol and acetonitrile were of HPLC grade and the others were of analytical grade. Sodium chloride, sodium hydroxide, sodium phosphate monobasic dihydrate and ammonium acetate were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). High-purity deionized water was used for preparation of aqueous solution and mobile phase.

All laboratory containers were made up of glass or Teflon and thoroughly cleaned by soaking in nitric acid (10%, v/v) for at least 24 h. Prior to use, all acid-washed ware were rinsed with high-purity deionized water. All solutions used in this study were filtrated through a polytetrafluoroethylene (PTFE)-made filter (0.45 μm, Tianjin Jinteng Instrument Factory, Tianjin, China) to discard granule.

2.2. Apparatus

The HPLC system (Hitachi, Hitachi High-Technological Corporation, Tokyo, Japan) was equipped with a Model L-2130 pump, an on-line solvent vacuum degasser, an auto sampler with 20 μL injection loop, and an L-2400 UV detector. A T2000P software was used to record chromatograms and calculate peak area. Chromatographic separations were carried out on a Symmetry® C_8 column packed with 5.0 μm particle size of dimethyloctylsilyl bounded amorphous silica (3.9 mm × 150 mm i.d., Waters, Ireland). The mobile phase was the mixture of ammonium acetate (0.03 mol L^{-1}, pH 5.5)–acetonitrile (60:40, v/v), delivered isocratically to the column at the flow rate of 1.0 mL min^{-1}. The column temperature was 25 °C. The detection wavelength was 238 nm.

An UV-756 MC Spectrophotometer (Shanghai Precision & Scientific Instrument Co., LTD., Shanghai, China) was used to obtain the absorption maxima of all analytes. The pH values were controlled with a Delta 320 pH meter (Mettler Toledo Instruments Co. Ltd., Shanghai, China) supplied with a combined electrode.

2.3. Standard solutions and calibration curves

Stock solutions (1.000 mg mL^{-1}) of amitryptiline, clomipramine and thioridazine were prepared by dissolving appropriate amount of their solid in acetonitrile, respectively. All the stock solutions were stored at 4 °C. Working solutions containing all the target drugs were prepared daily by dilution of the stocks with high-purity deionized water. Aqueous standard solutions were used to modify the separation/preconcentration conditions of DLLME.

The quantitative analysis was performed by the external standard method. A series of urine standards, prepared by diluting appropriate aliquots of the stock solution with drug-free urine at desired pH values, were subjected to the optimal DLLME procedure. The calibration curve for each drug was obtained by simple linear regression of each drug's concentration *versus* its peak area, and the concentration of analyte in sample was calculated based on the calibration curve.

The blank urine sample without any species of interest was used as the blank solution. The blank values for amitryptiline, clomipramine and thioridazine were determined after the blank solution was subjected to the procedure described for sample. The actual concentrations of the analytes were obtained after blank subtraction.

2.4. DLLME procedure

A 5.00 mL of aqueous sample solution containing the target analytes was placed in a 10 mL screw cap glass test tube. After rapidly and vigorously injecting 0.50 mL of acetonitrile (as disperser solvent) containing 20 μL of tetrachloride (as extraction solvent) into a sample solution using a 1.00 mL syringe, immediately, a cloudy solution was formed in the test tube (the cloudy state was stable for at least 2 h) and then the mixture was gently shaken. The separation of the phases was achieved by centrifugation at 4000 rpm for 3 min. After that process, different phenomenon was observed between aqueous standards and urine samples: For aqueous standards, a small droplet of carbon tetrachloride was sedimented in the bottom of the conical test tube [15–22]. But for urine sample, white lipidic solid was sedimented in the bottom of the conical test tube, probably due to the co-sedimentation of the matrixes (such as carbamide and uric acid) in urine at high pH values. After slowly discarding the aqueous solution, the resulting droplet and lipidic solid were dissolved to 200 μL with acetonitrile and then fil-

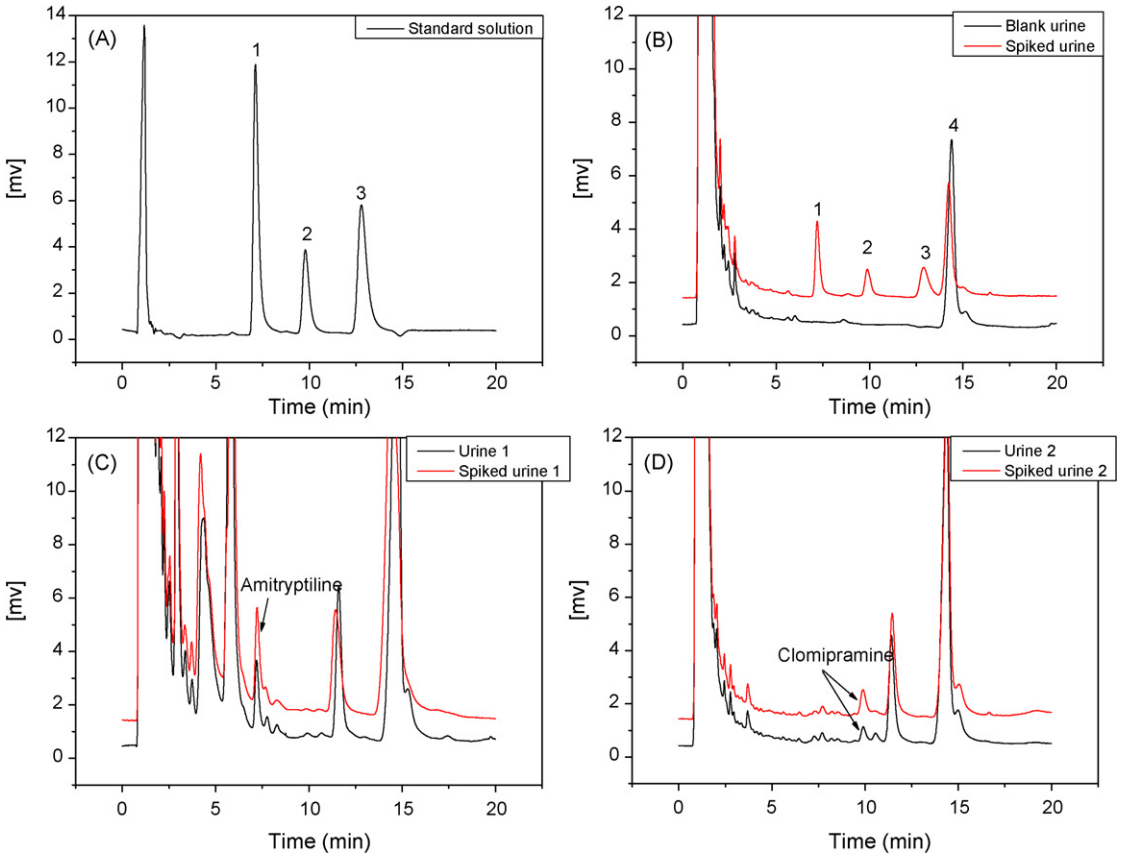

Fig. 1. Typical HPLC chromatograms of the target drugs for standard solution (A) without extraction, blank human urine (B), urine 1 (C) and 2 (D) after extraction using DLLME. Peaks 1–4 are amitryptiline, clomipramine, thioridazine and carbon tetrachloride, respectively. The concentrations of analytes in standard solution and spiked blank urine sample were 5.000 and 0.060 μg mL^{-1}, respectively. Urine 1 and 2 were from two patients under treatment with some psychotropic drugs including amitryptiline and clomipramine, respectively, and the concentrations of amitryptiline and clomipramine spiked in urine 1 and 2 were 0.025 μg mL^{-1}, respectively. DLLME conditions: sample volume and its pH values: 5.00 mL, 10.0; disperser solvent and its volume: acetonitrile, 0.50 mL; extraction solvent and its volume: CCl_4, 20 μL. Chromatographic conditions are described in Section 2.2.

trated through a 0.45 μm membrane to discard the white floccule in the extract of urine. Finally, appropriate volume of extracts was withdrawn into a microsyringe and then injected into the HPLC for subsequent analysis.

2.5. Sample preparation

Drug-free urine samples were provided by healthy volunteers not exposed to any drug for at least 2 months. Two urine samples were kindly provided by one female and male volunteer in our lab, respectively. In addition, two real urine samples collected from two female patients following a course of treatment with some psychotropic drugs including amitryptiline and clomipramine, respectively, were kindly provided by the psychiatric clinic of Wuhan Mental Hospital. The urine samples were respectively collected and stored in PTFE flasks at −20 °C until analysis.

The frozen urine samples were thawed at room temperature and centrifuged for 15 min at 4000 rpm. The supernatants were transferred to clean glass tubes and filtrated through a 0.45 μm filter. The resulting solutions were adjusted to pH 10.0 with 10 mol L^{-1} of NaOH solution and then subjected to the DLLME process.

3. Results and discussion

3.1. Optimization of HPLC-VU system

On the basis of the absorption maxima of amitryptiline, clomipramine and thioridazine in UV spectra acquired by UV-756 MC Spectrophotometer, the monitoring wavelength was set at 238 nm [4]. In this work, a C_8 column was used, and different elution conditions with methanol-water, acetonitrile–water and different concentrations of sodium phosphate monobasic dehydrate or ammonium acetate in water were investigated. The results indicated that mobile phase with ammonium acetate (0.03 mol L^{-1}, pH 5.5)–acetonitrile (60:40, v/v) had the best resolution factors (>1.5) for all targets indicating an excellent separation. Fig. 1A shows a typical chromatogram of standard solution. As can be seen, the retention times of target psychotropic drugs are 7.1, 9.8 and 12.8 min for amitryptiline, clomipramine and thioridazine, respectively. In order to assess the system suitability, the precisions of retention time and peak area were examined by evaluating the intra- and inter-day precisions of injection with standard mixture solutions of the target analytes at low, medium and high concentration levels on one day (each six measurements) and on three continuous days (each three measurements a day), respectively. The results indicated that the RSD values of the peak area and retention time were less than 3.0 and 0.5%, respectively.

3.2. Optimization of DLLME system

3.2.1. Extraction solvent and its volume

Choosing an appropriate extraction solvent is a significant consideration for extraction capability of the target analytes in DLLME. In this case, a satisfactory extraction solvent is required to fulfill the following functions: higher density than water, low solubility in water, non-volatile during extraction and good HPLC behav-

Table 1
Behaviors of extraction solvent in DLLME (mean ± SD, $n = 3$).

Analyte[a]	CCl_4 peak area	C_6H_5Cl peak area	$CHCl_3$ and CH_2Cl_2
Amitryptiline	$3.909 \times 10^5 \pm 1.945 \times 10^4$	$4.045 \times 10^5 \pm 2.137 \times 10^4$	No cloudy state and no sedimented droplet found
Clomipramine	$3.465 \times 10^5 \pm 1.524 \times 10^4$	$3.507 \times 10^5 \pm 2.035 \times 10^4$	
Thioridazine	$4.045 \times 10^5 \pm 1.907 \times 10^4$	–[b]	

[a] The concentrations of amitryptiline, clomipramine and thioridazine were 0.400, 0.900 and 0.500 μg mL^{-1}, respectively.
[b] The peak of thioridazine was overlapped by the peak of C_6H_5Cl.

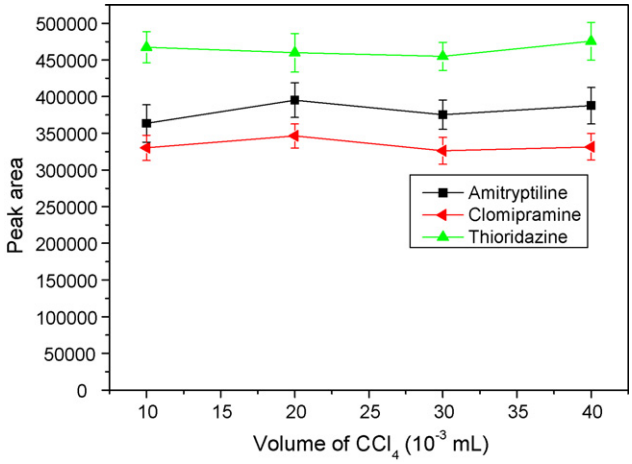

Fig. 2. Effect of extraction solvent (CCl_4) volume on extraction efficiency. Concentration of amitryptiline, clomipramine and thioridazine: 0.400, 0.900 and 0.500 μg mL^{-1}; sample volume: 5.00 mL; disperser solvent and its volume: acetonitrile, 0.50 mL; extraction solvent: CCl_4. Chromatographic conditions are described in Section 2.2.

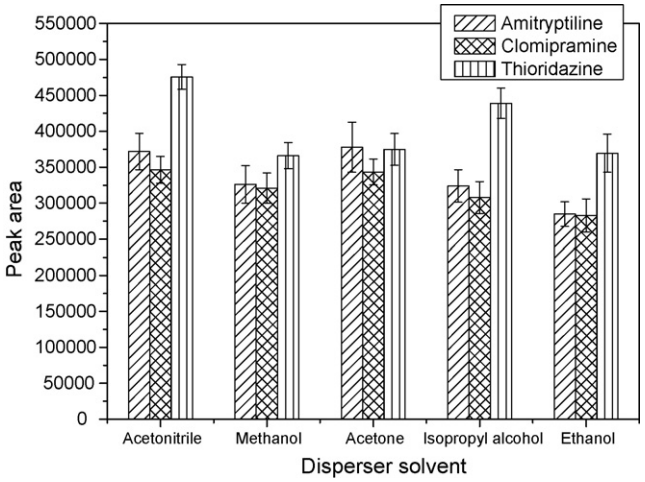

Fig. 3. Effect of disperser solvent type on DLLME. Concentration of amitryptiline, clomipramine and thioridazine: 0.400, 0.900 and 0.500 μg mL^{-1}; sample volume and its pH values: 5.00 mL, 10.0; disperser solvent and its volume: acetonitrile, methanol, acetone, isopropylalcohol and ethanol, 0.50 mL; extraction solvent and its volume: CCl_4, 20 μL. Chromatographic conditions are described in Section 2.2.

ior. Hence, several organic reagents including dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), and chlorobenzene (C_6H_5Cl) were investigated and their density values are 1.32 (CH_2Cl_2), 1.47 ($CHCl_3$), 1.59 (CCl_4), and 1.11 g mL^{-1} (C_6H_5Cl), respectively. The extraction efficiency was studied by using 0.50 mL acetonitrile containing 20 μL of the above described extraction solvent to extract the standard solutions (pH 10.0). The results shown in Table 1 indicated that when CH_2Cl_2 and $CHCl_3$ were used as extraction solvent, no cloudy state was observed and also no sedimented droplet of extract was found on the bottom of the tube after centrifuging, probably due to the high solubility of these solvent in water. Whereas, when use CCl_4 and C_6H_5Cl as extraction solvent, the cloudy state was formed and a sedimented droplet of extract was obtained on the bottom of test tube after centrifuging. After HPLC-UV analysis of the resulting extracts, it was found that similar extraction efficiency was obtained for amitryptiline and clomipramine when use CCl_4 and C_6H_5Cl as extract solvent, but under the optimal chromatographic conditions, the peak of thioridazine was partially overlapped by the peak of C_6H_5Cl. Thus, CCl_4 was selected as extraction solvent.

In order to optimize the extraction solvent volume, different volumes (10, 20, 30, 40 μL) of CCl_4 were added to 0.50 mL of acetonitrile and the resulting mixtures were subjected to the same DLLME procedures. As can be seen from Fig. 2, the signal intensity of the target analytes kept consistent at the whole tested range. Therefore, in the present work, the volume 20 μL was selected as optimal extraction volume of CCl_4.

3.2.2. Disperser solvent and its volume

In DLLME, an appropriate disperser solvent should be miscible with both extraction solvent and aqueous sample. In order to seek the most suitable disperser solvent, five kinds of disperser solvents (acetonitrile, methanol, acetone, isopropylalcohol and ethanol) were studied. As can be seen from Fig. 3, the maximum peak area was obtained by using acetonitrile as dispersive solvent. That is due to the synergic effect of good compatibility of acetonitrile with aqueous solution and low distributive ratio of analytes in the mixed solution of acetonitrile and water. Therefore, acetonitrile was selected as disperser solvent in the following experiment.

The influence of the volume of acetonitrile on the extraction efficiency of amitryptiline, clomipramine and thioridazine was also examined. As can be seen in Fig. 4, at first, the extraction efficiency increased and then decreased with the increase in the volume of

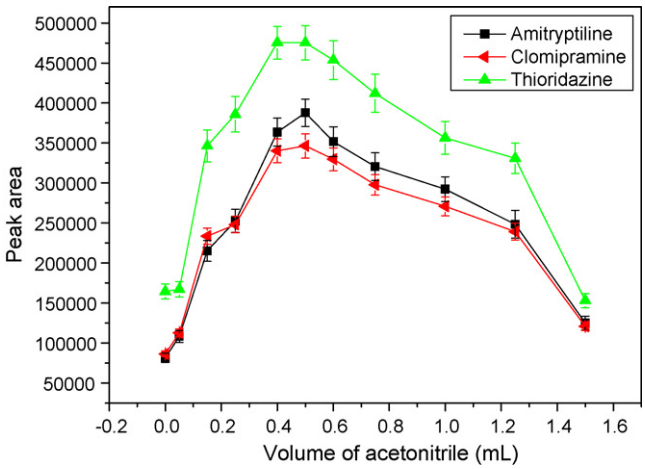

Fig. 4. Effect of disperser solvent (acetonitrile) volume on DLLME. drugs. Concentration of amitryptiline, clomipramine and thioridazine: 0.400, 0.900 and 0.500 μg mL^{-1}; sample volume and its pH values: 5.00 mL, 10.0; disperser solvent: acetonitrile; extraction solvent and its volume: CCl_4, 20 μL. Chromatographic conditions are described in Section 2.2.

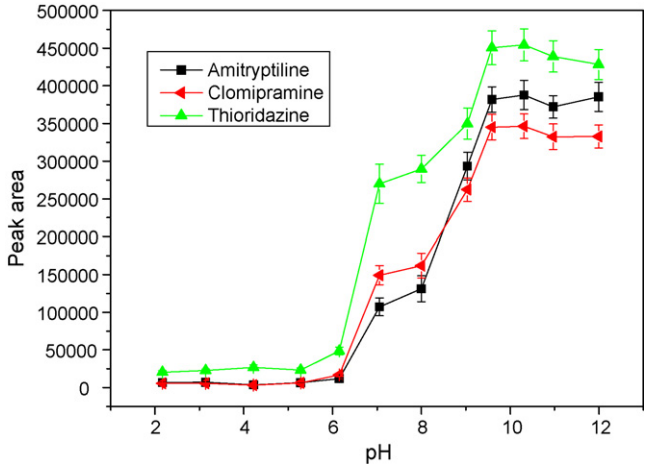

Fig. 5. Effect of pH values of aqueous solution on DLLME. Concentration of amitryptiline, clomipramine and thioridazine: 0.400, 0.900 and 0.500 μg mL^{-1}; sample volume: 5.00 mL; disperser solvent and its volume: acetonitrile, 0.50 mL; extraction solvent and its volume: CCl_4, 20 μL. Chromatographic conditions are described in Section 2.2.

acetonitrile. It seems that, at a lower volume of acetonitrile consumption, cloudy state was not formed well and the extract solvent of CCl_4 could not be well dispersed among the aqueous solution in the form of very little droplet, which resulted in poor extraction efficiency. Whereas, at a high volume of acetonitrile consumption, the solubilities of the target analytes and the extraction solvent (CCl_4) in water increased accordingly, thereby, the extraction efficiency also decreased. In subsequent study, 0.50 mL of acetonitrile was chosen as optimal dispersive volume.

3.2.3. Sample pH

It is well known that the pH of the sample solution was one of the important factors affecting the states of complexes (as ions or neutral forms). Fig. 5 shows the effect of pH on the peak area of the target analytes. As can be seen, the signal intensities of amitryptiline, clomipramine and thioridazine improved with the increasing of pH from 2.0 to 9.5, and then remained lightly constant in pH 9.5–12.0. This can be explained by the following reasons: Analytes in neutral forms are much easer to be extracted by extraction solvent than those in ion forms due to their strong affinity. According to literatures [23,24], the pK_a values of amitryptiline, clomipramine and thioridazine are 9.4, 9.4 and 9.5, respectively. Hence, when the pH of the aqueous was higher than the pK_a values of the analytes, the analytes are neutral forms in aqueous solution which have a greater tendency to be extracted into the extract solvent. Accordingly, the pH of samples was controlled at 10.0 for subsequent study.

3.2.4. Extraction time and centrifugation time

In DLLME, extraction time was defined as the time interval between the injection mixture of disperser solvent (acetonitrile) and extraction solvent (CCl_4), and before centrifugation [20]. To investigate the effect of extraction time on the extraction efficiency of target analytes, the extraction time of 0, 1, 2, 4 and 6 min were studied. The results indicated no obvious influence of extraction time on extraction efficiency. This can be explained as follows: After injecting the mixture of disperser solvent (acetonitrile) and extraction solvent (CCl_4), numerous small droplets of extract were instantaneously dispersed among aqueous solution as cloudy phase, indicating the infinitely large interface between the extraction solvent and the aqueous phase. Therefore, quick equilibrium was achieved due to the fast transition of analytes from aqueous phase to extraction solvent.

Table 2
Analytical performance of HPLC-UV of amitryptiline, clomipramine, and thioridazine on the C_8 column.

Analyte	Calibration curve[a]	R^2	Linear range (μg mL^{-1})	RSD (%)[b]		This work					Reference[c]	
				Repeatability	Reproducibility	EF[d]	LOD (ng mL^{-1}) Equipment	LOD (ng mL^{-1}) Method	LOQ (ng mL^{-1}) Equipment	LOQ (ng mL^{-1}) Method	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Amitryptiline	$Y = 9.808 \times 10^5X + 1.820 \times 10^4$	0.9998	0.020–3.000	4.2	4.5	24.1	68	3	205	10	0.5 [7][e]; 22.5 [4][f]; 20.1 [6][g]; 5 [14][h]	1.7 [7][e]; 70 [4][f]; 67 [6][g]; 15 [14][h]
Clomipramine	$Y = 3.666 \times 10^5X + 1.398 \times 10^4$	0.9992	0.040–6.000	3.7	3.8	23.7	149	7	447	21	25 [4][f]; 18.1 [6][g]	75 [4][f]; 59 [6][g]; 10 [14][h]
Thioridazine	$Y = 7.255 \times 10^5X + 4.501 \times 10^4$	0.9988	0.040–3.000	4.8	4.3	23.5	165	8	495	25	5.5 [5][i]	4.1 [26][j]

[a] Y, peak area; X, concentration of analytes expressed in μg mL^{-1}.
[b] C = 0.100 μg mL^{-1}, n = 9.
[c] Different sample preparation techniques combined with HPLC separation.
[d] Enrich factors obtained by comparing the slope of the calibration graph with/without preconcentration.
[e] Hollow fiber-based liquid phase microextraction.
[f] SPE using Lichrolut RP-18 Merck cartridges.
[g] SPE using multiwalled carbon nanotubes.
[h] LLE.
[i] SPE using Sep-Pak C_{18} cartridges.
[j] LLE.

Table 3
Analytical results of amitryptiline, clomipramine and thioridazine in two urine samples[a] (mean ± SD, $n = 3$).

Analyte	Added ($\mu g\,mL^{-1}$)	Urine M[b]		Urine F[c]	
		Found ($\mu g\,mL^{-1}$)	Recovery (%)	Found ($\mu g\,mL^{-1}$)	Recovery (%)
Amitryptiline	0	ND[d]		ND	
	0.040	0.041 ± 0.003	102	0.038 ± 0.002	95
	0.080	0.075 ± 0.004	94	0.076 ± 0.004	95
	0.160	0.147 ± 0.010	92	0.154 ± 0.007	96
Clomipramine	0	ND		ND	
	0.090	0.089 ± 0.010	99	0.095 ± 0.009	106
	0.180	0.187 ± 0.012	104	0.185 ± 0.008	103
	0.360	0.341 ± 0.023	95	0.349 ± 0.025	97
Thioridazine	0	ND		ND	
	0.050	0.047 ± 0.003	94	0.049 ± 0.004	98
	0.100	0.096 ± 0.005	96	0.101 ± 0.006	101
	0.200	0.191 ± 0.012	96	0.189 ± 0.014	94

[a] From our lab.
[b] Male volunteer.
[c] Female volunteer.
[d] Not determined.

Keeping the rotation speed at 4000 rpm, the centrifugation time (1, 2, 3, 4 and 5 min) was also studied. It was found that after a shorter centrifugation time (2 min), the maximum analytical signals for the target analytes were observed and then kept constant with the further increase of the centrifugation time. Thus the extraction time for subsequent analysis was fixed at 3 min.

3.2.5. Ion strength

The ion strength on extraction of target drugs was investigated by adding different amount of NaCl in the range of 0–10.0% (w/v). It was found that no obvious interference on extraction efficiency of the target drugs was observed after adding different amount of NaCl (1.0–10.0%, m/v). The results indicated the proposed method may be in particular suitable for the analysis of some samples with high concentration of salt such as urine.

3.2.6. Effect of urine matrix

The complex matrix in urine sample would cause the negative effect on the recovery of analytes under ordinary conditions, one main way was to dilute the urine samples at a risk of further decreasing the analytical sensitivity of analytes [4,6]. In order to validate the applicability of the proposed sample preparation technique for real biological sample matrix, the optimal conditions previously described were evaluated by comparing the analytical signals of the target drugs in blank urine samples and aqueous standards both spiked with same concentrations of the analytes. The experimental results indicated that no difference of analytical signals of target drugs was observed between aqueous standards and urine/diluted urine samples, which means that DLLME has high ability of resisting the interference of urine matrix. In view of the above facts, the urine sample adjusted to the desired pH 10.0 was directly used for DLLME procedure to obtain higher enrichment factor.

3.3. Recovery test

Under the optimal DLLME conditions, the absolute recovery of the target drugs from urine matrices was assessed by comparing the peak areas of extracted samples of spiked matrices *versus* non-extracted standards at three concentration levels. Mean recoveries (%) were 96, 97 and 101 for amitryptiline, clomipramine and thioridazine, respectively. The results indicated that quantitative extraction of the target drugs from urine samples was achieved by DLLME.

3.4. Stability test

In order to study the stability of target drugs in urine, a series of spiked urine samples with 0.200 $\mu g\,mL^{-1}$ of the target drugs were prepared by adding appropriate amounts of the target drugs into a drug-free urine sample provided by a health volunteer and then stored at −20 °C. Long-term stability was assessed one by one week during storage for one month, and the experimental results indicated that the recoveries of the target drugs remained constant during the test month. Thus the stability was for at least one month, similar result was observed in literature [4]. In addition, the stock solutions of amitryptiline, clomipramine and thioridazine stored at 4 °C keep unchanged for 3 months.

3.5. Validation of the method

3.5.1. Analytical performance

Table 2 summarizes the linearity and sensitivity of the proposed method for the target drugs. The calibration curves were constructed by analyzing urine standards containing different concentrations of the target drugs previously treated by the optimized DLLME procedure. Limit of detection (LOD) and quantification (LOQ) for each analyte were calculated based on the equation $LOD = 3.3\sigma/S$, $LOQ = 10\sigma/S$, respectively, where σ is the standard deviation (SD, $n = 3$) of the intercept of the regression lines and S is slope of the calibration graph [4,25]. The enrich factor (EF) of analyte was obtained by comparing the slope of the calibration graph with/without preconcentration. The repeatability and reproducibility of the method were the relative standard deviations (RSDs) for nine replicate determinations of a urine sample at 0.100 $\mu g\,mL^{-1}$ level of the analytes on one day and on three different days (three time a day), respectively. Additionally, as a comparison, the LODs or LOQs of amitryptiline, clomipramine, and thioridazine for equipment and those obtained in other works based on different sample preparation techniques combined with HPLC have also been listed in Table 2. As can be seen, the LODs and LOQs of this method are one order lower than those of equipment. Additionally, although the LOD or LOQ values obtained by this work are higher than those obtained in literature [7,26], they are comparable with those reported by literature [5,14] and even lower than those in literature [4,6].

Typical chromatograms of blank urine and spiked urine samples (after DLLME) are given in Fig. 1B.

3.5.2. Application

The proposed method was firstly applied to determine the concentrations of the target drugs in two real urine samples, provide by one male and female volunteer in our lab, respectively, and the obtained results are summarized in Table 3. As can be seen, there was no analytes of interest found in real urine samples, and the obtained results for spiked urine samples are in good agreement with the respected values with satisfactory recovery range of 92–106%.

Two actual urine samples collected from two female patients following a course of treatment with some psychotropic drugs combinations including amitryptiline and clomipramine, respectively, were subjected to the proposed procedure of DLLME, and analyzed in triplicate. It was found that the concentration of amitryptiline and clomipramine in the urine samples were 0.027 ± 0.001 and $0.019 \pm 0.002\ \mu g\ mL^{-1}$ (As the concentration of clomipramine in urine sample was lower than the LOD, its value was estimated by extrapolation of the linear range), respectively, indicating a good correlation with the drug and dose delivered to the patient. In order to validate the method, $0.025\ \mu g\ mL^{-1}$ of amitryptiline and clomipramine were spiked into the urine samples, respectively, and their concentrations obtained in spiked urine samples were 0.051 ± 0.003 and $0.045 \pm 0.002\ \mu g\ mL^{-1}$ with satisfactory recoveries (96% and 104%, respectively). The chromatograms of the above mentioned urine samples are given in Fig. 1C and D, respectively. As can been seen, the presence of major endogenous components, coexisting drugs and their metabolites in urine samples has no obvious influence on the determination of the target analytes under the selected conditions and the proposed method has a good selectivity for the analysis of analytes.

4. Conclusion

A new method of dispersive liquid–liquid microextraction combined with HPLC-UV has been described for the determination of amitryptiline, clomipramine, and thioridazine in urine samples. In conventional application, DLLME was widely used for the analysis of organic pollutants in environmental water samples with relative simple matrices. In this method, DLLME was firstly used for the analysis of several drugs in urine samples with complex matrices. Compared with other conventional modes (e.g., liquid phase extraction and solid-phase extraction) for the preparation of urine samples, the presented DLLME method provided numerous merits such as simplicity, easy/fast operation, high enrichment factor and lower detection limit. In addition, DLLME is seemed to have vast potential of application for the analysis of other drugs in urine sample, as a useful tool in human medicine for estimating and personalizing the effective drug dose in patients in clinical situations.

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