Determination of 13 Organic Toxicants in Human Blood by Liquid-Liquid Extraction Coupling High-Performance Liquid Chromatography Tandem Mass Spectrometry

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Pesticides and antidepressants are frequently misused in drug-facilitated crime because of their toxicological effect and easy-availability. Therefore, it is essential for the development of a simple and reliable method for the determination of these organic toxicants in biological fluids. Here, we report on an applicable method by the combination of optimized liquid-liquid extraction (LLE) procedure and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to identify and quantify dimethoate, omethoate, dichlorvos, carbofuran, fenpropathrin, diazepam, estazolam, alprazolam, triazolam, chlorpromazine, phenergan, barbitone and phenobarbital in human blood. The method demonstrated a linear calibration curve in range of 20 – 500 µg/L ($r > 0.994$). The accuracy evaluated by recovery spiked at three different concentrations (50, 100 and 200 µg/L) was in the range of 58.8 – 83.1% with a relative standard deviations (RSD) of 3.7 – 7.4%. The limits of quantification ranged over 6.7 – 33.3 µg/L. This method was proved to be simple and reliable, and was thus successfully applied to forensic toxicology.

Keywords HPLC/MS/MS, liquid-liquid extraction, pesticides, antidepressants, forensic toxicology, whole blood

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Introduction

As common organic toxicants, pesticides (PETs) and antidepressants (ATDs) always pose a threat to human being's life once misused. PETs are extensively used in most countries due to their effective protection of agricultural and horticultural crops against damage as well as the effect of eradication of household pests.¹⁻⁴ It is a common occurrence of fatal poisoning with PETs due to accidental ingestion and ingestion for suicidal or homicidal purposes. It is reported that poisoning cases with pesticides have been considered to be important causes of morbidity and mortality in all parts of the world.⁵⁻¹⁰ ATDs, as the first choice in the treatment of depressive illness, are becoming increasingly used due to the rapid growth in population of depression-affected people worldwide.¹¹⁻¹⁴ Meanwhile, the abuse of ATDs has become a significant problem in societies across the world because of their easy availability and toxicological effect.¹⁵⁻¹⁹ Over the past decades, forensic cases related to ATDs abuse was quite common, such as driving under the influence of drugs, violent crime, drug-facilitated sexual assault and other cases of sudden or violent deaths.²⁰⁻²² Therefore, ATDs and/or PETs are some of the most frequently encountered compounds in forensic and clinical cases. Up to date, numerous analytical methods have been developed, but most of them are limited to quantify either pesticides or antidepressants in biological samples.^{23,24} However, biological samples include, but are not limited, to one kind of organic toxicants instead, both pesticides and antidepressants likely exist in the same samples. For example, omethoate (OMT) and

diazepam (DZP), fenpropathrin (FEN) and DZP, dichlorvos (DDVP) and phenobarbital (PNB), DDVP and barbitone (BBT) had been confirmed in the same blood samples in our laboratory analysis during the past two years. What is more, the simultaneous analytical method for the detection of toxicants with different characteristics is urgently needed with respect to a rapid determination of unknown toxicants in various samples.²⁵ Therefore, the quantification of pesticides and antidepressants in the same method are indispensable for poisoning patient treatment and/or providing judicial organs with useful clues for investigating organic toxicant poisoning-related cases.

Each kind of PETs and ATDs tend to demonstrate different chemophysical properties from others due to the differences in their chemical structures, polarities, acidity and alkalinity, solubility in various solvents *etc.* For example, organophosphorus, carbamate and synthetic pyrethroid pesticides are easily soluble in organic solvents (*e.g.*, dichloromethane, diethyl ether and ethyl acetate) but poorly soluble in water due to their low polarity. Moreover, some of them can be decomposed in a basic aqueous solution. Benzodiazepines and phenothiazines have low polarities and weak alkalinities, while benzodiazepines show weak acidities. Like PETs, most of them are soluble in an organic solvent, but poorly soluble in water. Therefore, specific solvents and pH conditions were required during the pretreatment of samples containing different kinds of organic toxicants. Up to date, many specific methods for determination of each kind of organic toxicants have been developed based on analysis of chemophysical properties of target analytes. However, these methods usually require multiple pretreatments and different analytical methods when the samples contain more than one kind of analytes, which make procedures complicated.²⁶⁻³²

Table 1 Selected analytes in this study

Category	Analyte	Abbreviation	Chemical structure	CAS registry No.	Molecular weight	Molecular formula
Pesticide	Dimethoate	DMT		60-51-5	229.3	C ₅ H ₁₂ NO ₃ PS
	Omethoate	OMT		1113-02-6	213.2	C ₅ H ₁₂ NO ₄ PS
	Dichlorvos	DDVP		62-73-7	221.0	C ₄ H ₇ Cl ₂ O ₄ P
	Carbofuran	CBF		1563-66-2	221.3	C ₁₂ H ₁₃ NO ₃
	Fenpropathrin	FEN		64257-84-7	349.4	C ₂₂ H ₂₃ NO ₃
Antidepressant	Diazepam	DZP		439-14-5	284.7	C ₁₆ H ₁₃ ClN ₂ O
	Estazolam	EST		29975-16-4	294.7	C ₁₆ H ₁₁ ClN ₄
	Alprazolam	ALP		28981-97-7	308.8	C ₁₇ H ₁₃ ClN ₄
	Triazolam	TAL		28911-01-5	343.2	C ₁₇ H ₁₂ Cl ₂ N ₄
	Chlorpromazine	CLP		50-53-3	318.9	C ₁₇ H ₁₉ ClN ₂ S
	Phenergan	PNG		58-33-3	320.9	C ₁₇ H ₂₁ ClN ₂ S
	Barbitone	BBT		57-44-3	184.2	C ₈ H ₁₂ N ₂ O ₃
	Phenobarbital	PNB		50-06-6	232.2	C ₁₂ H ₁₂ N ₂ O ₃

Among existing pretreatment techniques, liquid-liquid extraction (LLE) is the most widely used as a routine method to pretreat various samples with complex matrices due to its efficiency of cleanup, enrichment, signal enhancement and without special equipments, though which suffers some drawbacks, such as

requirements of amounts of sample volumes, time consuming and tedious process and so on.³³ Especially, LLE for sample preparation was frequently employed to determine ATDs in brain tissue or blood due to clogging of sorbents during SPE.³⁴ Therefore, LLE is still a popular and efficient method for

pretreatment of biological samples, especially in the routine analysis.³⁵ Additionally, HPLC-MS/MS has been extensively applied in clinical and forensic toxicology work due to their high sensitivity, selectivity and ability to provide rich information on analytes.³⁶ In recent years, HPLC-MS/MS has been commonly employed in determination of various organic toxicants in different samples.³⁷⁻³⁹

In this work, dimethoate (DMT), OMT, DDVP, carbofuran (CBF) and FEN were chosen as target analytes to evaluate the efficiency of the developed method, because they have been frequently used in the human poisoning and/or suicide cases in our district, and even in some parts of China.⁴⁰⁻⁴² In addition, the chemical-physical properties of the chosen compounds varied over a wide range, but the suitability of the method can be well validated by selecting these compounds as the target analytes. Finally, DMT, OMT, DDVP, CBF and FEN are representative compounds of organophosphorus pesticides, while CBF and FEN are representative ones of carbamic acid esters and pyrethroids pesticides, respectively. DZP, estazolam (EST), alprazolam (ALP), triazolam (TAL), chlorpromazine (CLP), phenergan (PNG), BBT and PNB were chosen as target analytes in this work for a similar reason.

Under this background, a simple and reliable method was successfully developed through combination of optimized LLE and HPLC/MS/MS, which allows the rapid determination of organic toxicants with different kind of chemophysical properties including DMT, OMT, DDVP, CBF, FEN, DZP, EST, ALP, TAL, CLP, PNG, BBT and PNB, the chemical structures and other information of analytes were presented in Table 1. The sensitivity, linearity, accuracy and repeatability of this method were fully investigated. Moreover, the method was also applied to the analysis of whole blood samples from forensic investigations to verify its effectiveness. The results indicated that such a method allows the analysis of whole blood samples containing some of the studied drugs in routine laboratories.

Experimental

Reagents and chemicals

DMT, OMT, DDVP, CBF, FEN were purchased from Dr Ehrenstorfer (Augsburg, Germany) while DZP, EST, ALP, TAL, CLP, PNG, BBT, PNB were purchased from Sigma-Aldrich (St. Louis, USA), having purity higher than 99.5%. HPLC-grade methanol, and acetonitrile were obtained from TEDIA (OH, USA). All other chemicals were of analytical-grade in the highest purity available. Water was distilled and purified using a Millipore Milli-Q Plus system (Bedford, USA).

Apparatus

The chromatographic analysis was performed on a LC-20A liquid chromatography (Shimadzu, Japan)-API2000 tandem triple quadrupole mass spectrometer (AB Technologies, USA).

Preparation of stock solutions, working solutions, and quality control (QC) samples

Stock solutions of each analytes having a concentration of 1 mg/L were prepared in methanol and stored at 4°C in the absence of light. Mixed calibration standard solutions of thirteen analytes were obtained by serial appropriate dilution of aliquots of the stock solutions with methanol in order to yield the following concentrations: 10, 20, 50, 100, 200 and 500 µg/L. Working solutions of all analytes were freshly prepared each day. Quality control (QC) samples for all analytes were prepared by addition of appropriate amounts of the stock

solutions to 1 mL of analyte-free whole blood to make three different concentrations, namely, 50, 100 and 200 µg/L.

Sample collection and preparation

Analyte-free blood samples collected from healthy male adults were used to make QC and control samples; 10 mL of blood taken from the victims, necessary for method development and validation, were provided by Local Public Security Bureau.

Blank and QC were pretreated by following four procedures, whereas the real samples only pretreated by the total LLE procedure.

LLE under acidic condition (acidic LLE procedure). An accurately measured volume of 1 mL blood sample was placed in a 15-mL of centrifuge tube and diluted with 2 mL of Milli-Q water and then acidified with diluted hydrochloric acid (HCl) solution to give a final pH of 2. The tube was capped by the addition of 5 mL of an organic solvent (dichloromethane, ethyl acetate and diethyl ether) and submitted to vortex mixing for 5 min and then sonicated for another 5 min. After centrifugation at 9500 r/min for 5 min, the upper organic phase was transferred to conical tubes and separated. Then, the residue was further extracted by the same procedure again, and the organic phase was transferred to the same conical tubes. The combined organic phase was blown to dryness and the residue was dissolved in 0.5 mL of methanol and filtered with a Nylon filter (0.22 µm) for HPLC/MS/MS analysis.

LLE under neutral condition (neutral LLE procedure). This procedure is same to the acidic LLE procedure except for omission of the pH adjustment.

LLE under basic condition (basic LLE procedure). This procedure is the same as the acidic LLE procedure, except that the pH of the diluted blood sample was adjusted to 10 with a saturated sodium carbonate (Na₂CO₃).

LLE under neutral-basic-acidic condition (total LLE procedure). As for this procedure, 1 mL of blood sample was first pretreated with the neutral, and then basic (pH 10) and finally acidic (pH 2) extraction procedures and the sample was extracted only once at each step. After extraction, all organic phases were combined, and the subsequent processing procedures were the same as above mentioned ones.

Chromatographic conditions

Chromatographic conditions for PI mode. Gradient elution was performed on a separation column (Waters Atlantis TM dC18, 150 × 3.9 mm, 5.0 µm; Waters Technologies, USA). The mobile phase consisted of methanol (eluent A) and water containing 0.1% formic acid (eluent B). The gradient was programmed as follows: 0.0 – 3.0 min: gradient decreased from 80 to 5% eluent B; 3.0 – 10.1 min: gradient 5% eluent B; 10.0 – 10.1: gradient increased from 5 to 80% eluent B; 10.1 – 20.0 min: gradient 80% eluent B. The total analysis time was 20 min. The flow rate was set at 0.5 mL/min while the injection volume was 20 µL. Moreover, the temperature of the column was kept at 35°C.

Chromatographic conditions for NI mode. The same separation column employed as that in PI mode was used in this mode. The mobile phase consisted of acetonitrile (eluent A) and water (eluent B). The gradient was programmed as follows: 0.0 – 3.0 min: gradient decreased from 80 to 5% eluent B; 3.0 – 9.0 min: gradient 5% eluent B; 9.0 – 10.0: gradient increased from 5 to 80% eluent B; 10.0 – 20.0 min: gradient 80% eluent B. The parameters of the total analysis time, flow rate, injection volume and the temperature were the same as those in PI mode.

Mass spectrometric conditions. Analyses of analytes, except for

Table 2 MRM transitions per analyte (Q1: precursor ion mass, Q3: product ion masses) and respective settings for LC-MS/MS system (CE: collision energy, DP: declustering potential, EP: entrance potential, CEP: collision cell exit potential)

Analyte	Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	<i>t_R</i> / min	ESI mode	CE/V	DP/V	EP/V	CEP/V
DMT	230.0	198.9 ^a 124.9	6.21	+	10, 10	10, 10	13, 30	6, 6
OMT	214.0	182.9 ^a 154.9	5.07	+	15, 21	26, 26	5, 6	8, 9
DDVP	220.9	127.0 108.9 ^a	6.32	+	25, 29	33, 33	6, 6	5, 5
CBF	222.1	165.0 ^a 122.8	6.34	+	10, 10	30, 30	15, 25	5, 5
FEN	350.2	125.1 ^a 97.2	7.78	+	19, 40	22, 23	10, 9	7, 7
DZP	285.1	193.3 ^a 154.1	6.92	+	43, 36	44, 42	10, 10	10, 5
EST	295.1	267.0 ^a 204.9	6.52	+	61, 34	57, 57	11, 11	4, 4
ALP	309.1	281.0 ^a 205.1	6.64	+	10, 10	40, 40	10, 10	7, 6
TAL	343.3	314.6 ^a 307.8	6.51	+	38, 40	60, 70	10, 10	20, 20
CLP	319.1	246.0 86.1 ^a	5.83	+	32, 33	34, 36	8, 8	5, 5
PNG	285.2	198.1 86.1 ^a	5.64	+	25, 25	23, 23	6, 6	4, 5
BBT	182.9	140.0 ^a 84.9	5.15	–	–14, –16	–20, –20	–6, –6	–6, –5
PNB	230.9	187.9 85.0 ^a	5.61	–	–16, –12	–12, –13	–6, –7	–6, –15

a. Quantifier ion.

BBT and PNB, were conducted in positive electrospray ionization mode (ESI⁺) using multiple reaction monitoring (MRM). Nitrogen (N₂) was used as the sheath and auxiliary gas while helium (He) was used as the collision gas. The optimum conditions of the interface and MSD parameters were as follows: curtain gas of 20 psi, ion source gas 1 of 65 psi, ion source gas 2 of 70 psi, collision gas of 4, ion spray voltage of 5500 V and the interface temperature of 450°C. For the analysis of BBT and PNB, the MS parameters were operated in the negative multiple reactions monitoring scan mode (ESI[–]-MRM), and the condition was similar to ESI⁺-MRM, except that the voltage was set at –4200 V. The mass spectrometer was operated in the information-dependent acquisition (IDA) mode. All other settings were analyte-specific and were determined using Analyst software in the quantitative optimization mode. The MRM transitions per analytes, respective settings and retention times for both LC-MS/MS systems are summarized in Table 2.

Results and Discussion

Chromatography

In order to obtain good separation and sharp peaks, methanol and acetonitrile or mixture of acetonitrile and methanol as an organic phase and water with different modifiers, such as ammonium formate, acetic acid, and formic acid at percentages were chosen to optimize the chromatographic conditions. As a result, it was found that methanol/water on the optimized gradient mode resulted in intense peaks for DMT, OMT, DDVP, CBF, FEN DZP, EST, ALP, TAL, CLP and PNG in PI mode while ionization efficiency in NI mode for BBT and PNB was

Fig. 1 Multi-reaction monitoring (MRM) chromatograms for 13 analytes at 100 µg/L: (1) DMT, (2) OMT, (3) DDVP, (4) CBF, (5) FEN, (6) DZP, (7) EST, (8) ALP, (9) TAL, (10) CLP, (11) PNG, (12) BBT and (13) PNB.

better with acetonitrile than methanol because acetonitrile was a weaker proton donor than methanol.⁴³ Signal enhancement can be achieved through the utilization of buffer additives in the mobile phase, such as ammonium formate, acetic acid, and formic acid.^{44,45} In the current method, formic acid was selected as modifier because it gave the overall best results for DMT, OMT, DDVP, CBF, FEN DZP, EST, ALP, TAL, CLP and PNG in respect to sensitivity. Then, the addition of different percentages of formic acid was tested and the results indicated that 0.1% formic acid led to both abundant signal response and effective suppression of the tailing phenomena of these analytes. On the contrary, the signal of BBT and PNB obviously reduced when formic acid was added to the mobile phase due to the use of NI scan mode. Therefore, methanol/water with 0.1% formic acid was chosen as the mobile phase when ESI⁺ was employed and acetonitrile/water containing no additives was used in the ESI[–].

On this basis, conditions of gradient elution were optimized. After optimization, gradient elution and separation condition beginning with 80% H₂O and 20% organic phase was found to be favorable for reduction of the matrix interference mainly produced by ionization of the polar fractions in the blood samples due to the effective elution of these compounds made by the mobile phase. In addition, the employed elution conditions also contributed to improvements of the symmetry of the peaks as well as abundant signal response of the target analytes. Retention time of each analyte ranged between 5.07 and 7.78 min (detailed in Table 2) under the described chromatographic conditions. Representative MRM chromatograms of a 100 µg/L standard mixture of the analytes fitted in each retention time window under optimized conditions are illustrated in Fig. 1.

LC-ESI-MS/MS

MS parameters including collision energy, declustering potential, entrance potential and collision cell exit potential were optimized by manual injection of 20 µL of each analytes. The OMT, DMT, DDVP, CBF, FEN DZP, EST, ALP, TAL, CLP and PNG were sensitive in PI mode, whereas the analysis of the BBT and PNB was carried out in NI mode because they were not possible to be optimized in the PI mode according literatures.^{46,47}

In order to select precursor ions for each analyte, chromatograms were recorded in the full scan mode. In this work, protonated molecular ions for DMT, OMT, DDVP, CBF,

Fig. 2 Tautomerism in barbiturate structures, tautomers I and III are the *keto* tautomers, while II and IV are the *enol* tautomers.

FEN DZP, EST, ALP, TAL, CLP and PNG in the PI mode and deprotonated molecular ions for BBT and PNB in the NI modes were chosen as precursor ions, respectively. Once the precursors were selected, product ions were obtained by testing different values of the cone voltage and collision energies. The cone voltages and collision energies were selected according to the highest sensitivity of precursor ions and the strongest intensity of the fragment ions obtained, respectively.

When tuned with a single standard solution of each analytes in the Q1 full scan, protonated molecule $[M+H]^+$ were observed at $m/z = 214.0, 230.0, 220.9, 222.1, 350.2, 285.1, 295.1, 309.1, 343.3, 319.1$ and 285.2 for DMT, OMT, DDVP, CBF, FEN DZP, EST, ALP, TAL, CLP and PNG, whereas the deprotonated precursor $[M-H]^-$ ions were observed at $m/z = 182.9$ and 230.9 for BBT and PNB due to the loss of proton by their *enol* tautomers (Fig. 2), respectively. The most intense transitions were used as quantifiers while the others were used as qualifiers for the confirmation of the analytes. The precursors to the product ion transitions as quantifiers monitored for DMT, OMT, DDVP, CBF, FEN, DZP, EST, ALP, TAL, CLP, PNG, BBT and PNB were at $m/z = 230.0/198.9, 214.0/182.9, 220.9/108.8, 222.1/165.0, 350.2/125.1, 285.1/193.3, 295.1/267.0, 309.1/281.0, 343.3/314.6, 319.1/86.1, 285.2/86.1, 182.9/140.0, 230.9/85.0$, respectively. The optimization parameters, precursor and product ions were listed in Table 2.

Effect of extraction parameters on extraction efficiency

Effect of solvents on extraction efficiency. As for the extraction of common organic toxicants by LLE, dichloromethane, ethyl acetate, petroleum ether and diethyl ether are frequently employed. Biological samples generally contain grease and pigments that are common matrix interferents. Therefore, the coextraction of these fractions and analytes in samples should be avoided as far as possible. At the beginning of the optimization of the extraction method for the isolation of thirteen organic toxicants, blood samples spiked with all analytes were extracted by using different organic solvents, namely, dichloromethane, ethyl acetate and diethyl ether. The analytes were extracted by taking advantage of the aforementioned total extraction technique, which includes three extraction steps. The pH of the blood sample was ~ 8 for the first step, and the pH was adjusted to 10 and 2 for the second and the third extraction step, respectively. The recoveries (R) of thirteen analytes were calculated by the following equation:

$$R = \frac{C_{\text{deter}}}{C_{\text{theor}}} \times 100\% \quad (1)$$

where C_{deter} and C_{theor} mean concentration of target analyte

Table 3 Mean recoveries ($n = 6$) obtained for selected analytes spiked in blood samples (the analytes were extracted with dichloromethane, ethyl acetate and diethyl ether, respectively)

Analyte	Added concentration/ $\mu\text{g L}^{-1}$	Dichloro-methane		Ethyl acetate		Diethyl ether	
		Rec., %	RSD, %	Rec., %	RSD, %	Rec., %	RSD, %
DMT	500	74.8	6.3	76.5	6.1	66.4	4.3
OMT	500	75.6	6.5	70.5	5.6	52.3	5.1
DDVP	500	63.7	7.1	72.1	4.7	54.4	4.5
CBF	500	62.2	4.8	76.7	6.5	74.3	5.3
FEN	500	59.6	5.1	67.4	5.7	61.2	5.1
DZP	500	79.8	6.8	85.7	6.2	89.0	4.4
EST	500	76.7	5.3	76.6	5.2	78.3	5.5
ALP	500	75.5	4.5	77.4	6.4	70.8	4.7
TAL	500	56.4	5.2	67.0	3.9	57.2	5.8
CLP	500	69.2	4.3	79.4	5.4	74.1	4.7
PNG	500	75.2	5.4	87.4	6.6	59.3	5.7
BBT	500	59.5	6.9	76.1	7.1	85.6	6.1
PNB	500	63.3	7.4	75.0	5.8	79.8	7.5

The extraction procedure including three steps and the pH value was controlled at $\sim 8, 10, 2$ for the first, second and third step, respectively.

detected in the spiked samples and theoretical concentrations ($\mu\text{g/L}$), respectively. As shown in Table 3, recoveries of all analytes for dichloromethane, ethyl acetate and diethyl ether ranged from 56.4 to 79.8%, 67.4 to 85.7%, 52.3 to 89.0% with RSD of 4.3 – 7.4, 3.9 – 7.1 and 4.3 – 7.5, respectively. Results indicated that the best overall extraction efficiency for all analytes was achieved when ethyl acetate was used. Moreover, ethyl acetate has advantages of lower toxicity and volatility than other two organic solvents as well as sufficient ability to dissolving many compounds including pesticides, hypnotics and tetramine, therefore, ethyl acetate was chosen as the extraction solvent in work.

Effect of pH and extraction number on extraction efficiency.

The pH value plays an important role in most of the extraction procedures, due to which greatly affects the existing form of the analytes in samples. In order to obtain the optimal pH required for extraction, the extraction efficiency of thirteen analytes with concentration of $500 \mu\text{g/L}$ was investigated in the acidic (pH 2, acidified by diluted HCl), neutral (pH ~ 8) and basic (pH 10, alkalized by saturated Na_2CO_3 solution) conditions using ethyl acetate as the extracting solvent. In addition, the effect of the extraction number on the extraction efficiency was also explored. The results illustrated in Table 4 reveal that the extraction efficiency was obviously affected by the pH value; as for FEN, DDP, PNB, the extraction efficiency at pH 2 was higher than that at pH 10 due to easy decomposition of FEN in basic media and the acidic properties of BBT and PNB, which was demonstrated by their enol tautomers (shown in Fig. 2).⁴⁸ On the contrary, OMT, CBF, DZP, EST, ALP, TAL, CLP and PNG were extracted more efficiently at pH 10 due to the basic properties produced by existence of “N” atoms in their molecules, additionally, the extraction efficiency of thirteen analytes at pH 8 was either nearly equivalent to or lower than that in the acidic or basic conditions. The effect of the extraction number on the extraction efficiency is also shown in Table 4. As can be seen from Table 4, the extraction efficiency obviously increased as the extraction number was enhanced from 1 to 2 under different pH conditions. For instance, the extraction efficiency of DMT was increased from 59.4 to 70.2%, 62.1 to 68.9% and 63.5 to 72.4% when the extraction number increased

from 1 to 2 at pH 2, ~8 and 10. As for total LLE procedure, its extractive efficiency was superior or comparable to total extractive efficiency (the extraction number was 2) of acidic, neutral or basic extraction procedure. Therefore, the satisfactory extractive efficiency was achieved when the total extraction procedure was employed.

Effect of sonification time on extraction efficiency. The extraction time is one of the most important factors in most of extraction procedures. The effect of the sonication time on the extraction efficiency was investigated by varying the sonication time over the range of 2 – 10 min, and calculating the recovery by determining the spiked blood samples at a concentration of 500 µg/L for each analyte using ethyl acetate as the extracting solvent. The pH of the blood samples was ~8 for the first extraction step while the pH was adjusted to 10 and 2 for the second and the third extraction step, respectively (see *Sample collection and preparation*). The results of different sonification times *versus* the recovery ($n = 6$) are given in Table 5. It was found that the extraction recovery increased when the sonication time was increased from 2 to 5 min, after 5 min; the extraction recovery became nearly constant, and the reproducibility was

fairly good. Therefore, a 5-min sonication time was chosen as the working condition in this work.

Method validation

Linearity and LODs, and LOQs. The calibration curves were established by plotting the peak areas *versus* the concentrations. All analytes showed good linearity ($r > 0.994$) in a relatively wide test range (2 – 500 µg/L). The low limit of detection (LOD, obtained in whole blood) was denoted as the lowest concentration that the analytes could be detected with a signal-to-noise ratio being more than 3 ($S/N > 3$) while the limit of quantification (LOQ) was defined as the lowest concentration of the calibration curve at which the signal-to-noise ratio was more than 10 ($S/N > 10$). Based on the aforementioned definitions, the overall detection of limit and the quantification limit for DMT, OMT, DDVP, CBF, FEN, DZP, EST, ALP, TAL, CLP, PNG, BBT, PNB were 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 5, 10 and 6.7, 6.7, 6.7, 6.7, 6.7, 6.7, 6.7, 6.7, 6.7, 6.7, 16.7, 33.3 µg/L, respectively.

Extraction recovery, precision and accuracy. The extraction recoveries were evaluated by the determination of spiked blood samples ($n = 6$) at three quality control concentration levels (50, 100 and 200 µg/L) for each analyte. The precision (RSD) of the developed method was no more than 7.4%, and the accuracy (Recovery) ranged from 58.8 – 83.1%. It was noted that the recoveries of most analytes, except for BBT and PNB at low concentration (50 µg/L), were satisfactory. Therefore, this is a precise, accurate and credible method for the determination of the organic toxicants in blood samples.

Case examples. The practical application of the developed method using LLE-LC-MS/MS was validated at two examples.

Case #1: About 10 mL of a venous blood sample was taken from a victim of a drug-facilitated sexual assault. The 23-year-old female reported to the police that she had suffered a sexual assault after having coffee made by the suspects in a residential building. Blood was collected at the hospital 12 h after the crime.

Case #2: About 10 mL of heart blood was taken from a 46-year-old man. He was found dead in his room. Blood was collected at the hospital 24 h after his death.

Two collected blood samples were analyzed by the present methodology. Only ALP was found at concentration of 120 µg/L in the sample of Case #1 while DDVP and PNB at concentration of 83 and 157 µg/L were respectively confirmed

Table 4 Recoveries of analytes in blood under acidic, neutral, basic as well as total extraction conditions using ethyl acetate as extracting solvent

Analyte	Added concentration/ µg L ⁻¹	Acidic, %		Neutral, %		Basic, %		Total, %
		a	b	a	b	a	b	
DMT	500	59.4	70.2	63.5	72.4	62.1	68.9	68.5
OMT	500	53.9	70.5	56.1	67.1	68.2	74.5	69.4
DDVP	500	55.4	60.1	60.3	65.2	50.4	66.2	73.6
CBF	500	60.5	73.0	65.3	70.9	71.3	79.1	82.2
FEN	500	65.8	70.4	63.2	74.8	52.7	66.4	73.5
DZP	500	64.1	70.5	79.6	85.6	88.5	93.0	92.1
EST	500	62.0	69.4	63.2	78.3	80.0	88.4	87.2
ALP	500	58.4	67.9	76.4	82.5	74.0	82.9	85.4
TAL	500	58.0	67.5	70.8	76.9	73.0	79.6	81.2
CLP	500	62.1	69.0	60.6	73.0	74.5	81.0	83.6
PNG	500	57.2	62.5	72.6	79.8	78.5	80.1	80.7
BBT	500	78.3	83.4	69.5	74.6	61.2	66.4	82.1
PNB	500	72.4	79.8	62.5	67.9	59.6	63.8	78.6

a. Extraction number is 1.

b. Extraction number is 2.

Table 5 Effect of the sonication time on the average recovery ($n = 6$) of 13 analytes

Analyte	Added concentration/µg L ⁻¹	Recovery ^a , %	RSD ^a , %	Recovery ^b , %	RSD ^b , %	Recovery ^c , %	RSD ^c , %
DMT	500	52.9	5.3	56.1	4.7	58.2	7.3
OMT	500	60.6	4.9	73.4	5.2	76.3	6.9
DDVP	500	66.1	5.6	76.7	3.7	78.3	5.2
CBF	500	66.0	6.1	71.2	5.3	68.2	8.2
FEN	500	61.5	4.8	69.7	4.1	68.2	7.1
DZP	500	81.8	5.3	91.1	6.6	92.3	5.6
EST	500	80.1	6.3	82.4	4.7	88.6	6.4
ALP	500	73.7	5.1	77.3	6.1	81.7	7.5
TAL	500	59.5	5.7	66.4	5.6	70.8	4.1
CLP	500	71.5	4.5	70.4	6.3	73.8	5.7
PNG	500	64.8	5.3	78.0	6.8	81.5	5.2
BBT	500	66.3	6.4	72.1	7.6	73.5	8.7
PNB	500	72.1	6.9	77.3	5.1	79.1	7.4

The system was sonicated for (a) 2 min, (b) 5 min and (c) 10 min, respectively. The analytes were extracted from 1 mL of whole blood samples, which includes three steps and pH value was controlled at ~8, 10, 2 for the first, second and third step, respectively. Ethyl acetate was used as extracting solvent.

in the sample of Case #2. The results indicated that the developed method can be applied to toxicological analysis.

Conclusions

In this study, thirteen organic toxicants in human blood were efficiently extracted by a total LLE procedure, and were rapidly determined by HPLC/MS/MS. The developed method demonstrated wide linearity, relatively low LOD, good reproducibility and recovery of all target analytes with different chemophysical properties. In addition, the developed method was successfully applied to determine ALP, DDVP and PNB in real samples for forensic toxicology.

We expected that the developed method could be extended to determine compounds with the similar structures to those analyzed in this study in more biological samples including plasma, whole blood, oral fluids, hair and tissues, which are frequently encountered in forensic and clinical analysis.

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