Analytical Methods

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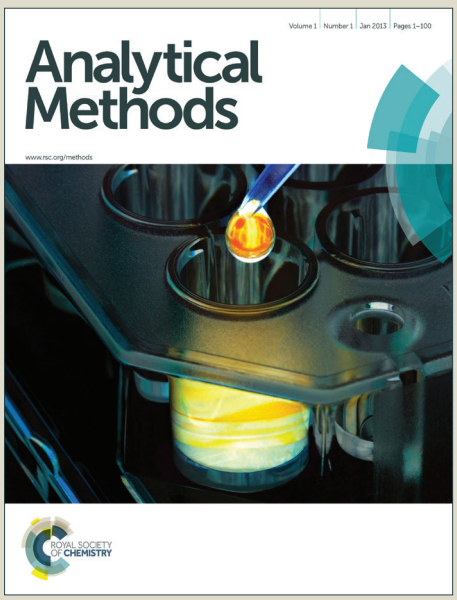

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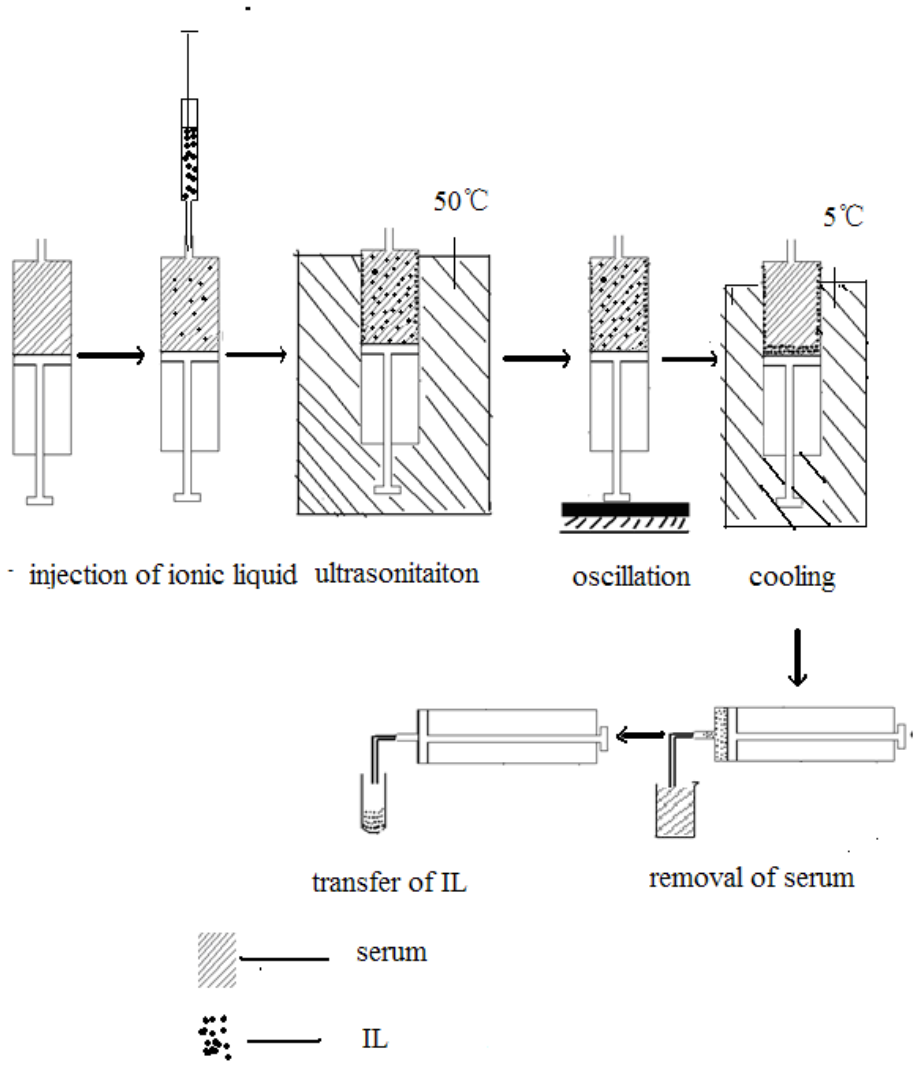

In - syringe ionic liquid dispersive liquid-liquid microextraction for the determination of sulfonamides in blood using high-performance liquid chromatography

Wei Yu[a] Zhongling Liu[b] Shusen Cui[a] Shaohua Zhang[c] Xiao Yang[b] Lei Lei[b] Hanqi Zhang[b] Aimin Yu[b,*]

[a] *Department of Hand Surgery, China-Japan Union Hospital, Jilin University, Xiantai Street 126, Changchun 130033, PR China*

[b] *College of Chemistry, Jilin University, Qianjin Street 2699, Changchun 130012, PR China*

[c] *Department of Rehabilitation, the General Hospital of FAW (the Fourth Hospital of Jilin University), Haoyue Road 3788, Changchun 130011, PR China*

*Corresponding author. Aimin Yu

Tel.: +86 431 85168399; fax: +86 431 85112355.

E-mail address: liuzhongling1988@163.com

Abstract

In - syringe ionic liquid dispersive liquid-liquid microextraction was developed and applied for the extraction of sulfonamides in blood. High-performance liquid chromatography was applied to the determination of the analytes. The parameters affecting the extraction efficiency, including volume of ionic liquid, salt concentration, pH value of system, extraction time and temperature, and cooling time were investigated. The limits of detection for sulfadiazine, sulfamethyldiazine, sulfamethizole, sulfachlorpyridazine, sulfamethoxazole and sulfisoxazole were 12.30, 2.22, 3.19, 1.67, 2.93 and 1.58 ng mL^{-1}， respectively. When the present method was applied to the analysis of blood samples, the recoveries of the analytes ranged from 87.0 to 115.3 % and relative standard deviations were lower than 7.2 %.

Key words: In - syringe ionic liquid dispersive liquid-liquid system; Blood; Sulfonamides; High-performance liquid chromatography.

1. Introduction

Sulfonamides (SAs) are synthetic antimicrobial medicines and frequently used for prevention or treatment diseases of animals in all the countries of the world [1]. The SAs are used in animal husbandry, aquaculture and also used as medicines to treat many kinds of infection [2]. The overuse of the medicines may lead to the contamination in livestock products, which can give rise to an increase in the antibiotic resistance of pathogenic bacteria [3]. Long-term use of SAs can result in serious side effects, such as emiction and hemopoiesis turbulence. To protect public health, many government authorities restrict the dose of SAs strictly. So it is of great importance to develop an effective method for determinating trace level SAs.

Therefore, there is a need for the development of simple rapid inexpensive method for determining SAs. At present, a number of analytical methods such as capillary electrophoresis (CE) [4,5], gas chromatography (GC) [6,7], and high performance liquid chromatography (HPLC) [8,9,10] have been applied for determination of SAs. The methods using HPLC coupled with various detection methods such as ultraviolet (UV) [11,12], photodiode array (PDA) [13], fluorescence [14] or mass spectroscopy (MS) [15] have received a lot of attention. Compared with other detection methods, when UV was applied, lower cost will be paid. Therefore, HPLC coupled with UV detection is most widely applied [11]. Solid-phase extraction (SPE) [16,17], solid-phase microextraction (SPME) [18,19], matrix solid-phase dispersion [20], liquid-liquid extraction (LLE) [21], liquid phase microextraction (LPME) [22,23] were commonly used as sample pretreatment techniques for the determination of SAs. However, LLE can lead to the consumption of a large amount of organic solvent. The precision obtained by LPME was relatively poor due to the manually handling of a small amount of extraction solvent.

The dispersive liquid-liquid microextraction (DLLME) was developed by Assadi and co-workers [24,25], and has attracted much attention in recent years for its superior advantages of high enrichment factor, perfect recovery, low cost, rapid and

easy operation [26]. The conventional IL-DLLME [27] is simple and no extra energy is required. The method is based on the simple mixture of the aqueous sample with the hydrophobic IL used as extraction solvent and the dispersive solvent. The mixture can be manually stirred. Temperature-controlled IL-DLLME [28] requires heating of the sample and the hydrophobic ILs is used as extraction solvent. The cooling of the extraction system is required before the phase separation. In the ultrasound-assisted [29] or microwave-assisted IL-DLLME [30], ultrasound and microwave are required, respectively, to facilitate dispersion of the hydrophobic IL into the sample solution and to ensure the complete extraction of the analytes. In in-situ IL-DLLME [31], the hydrophilic IL is used as extraction solvent. The anion exchange reagent is then added in the aqueous sample containing hydrophilic IL and the hydrophobic IL is formed. In Ionic liquid/ionic liquid dispersive liquid–liquid microextraction (IL/IL-DLLME) two kinds of nonvolatile ILs, hydrophobic IL and hydrophilic IL, were used as extraction solvent and dispersion solvent, respectively [32]. The method is based on the emulsification of hydrophobic IL in an aqueous sample. In the IL-DLLME methods mentioned above the separation between aqueous phase and IL phase is carried out by the centrifugation. IL-DLLME has been applied for extraction of polycyclic aromatic hydrocarbon [33], organophosphate esters [34], phthalate esters [35], anilines [36] and SAs [37].

In this work, in - syringe IL-DLLME was applied to the extraction of SAs in blood samples. Several factors affecting the extraction were evaluated. Some real samples were analyzed and the application of the present method in pharmacokinetic investigation was developed. Compared with IL-DLLME, The centrifugation for separating the ionic liquid from sample solution was not required and the operation was simpler.

2. Experimental

2.1. Reagents and chemicals

Sulfadiazine (SDZ), sulfamethyldiazine (SMZ), sulfamethizole (STZ), sulfachlorpyridazine (SCP), sulfamethoxazole (SMX) and sulfisoxazole (SSZ) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The purity of all the compounds was higher than 98%. The structures of these SAs are shown in Fig.1. Standard stock solutions for the SAs at the concentration level of 500 μg. mL^{-1} were prepared in acetonitrile containing 5.24 mmol L^{-1} acetic acid. The working solutions were obtained by diluting the stock solutions with pure water. 1-Hexyl-3-methylimidazoliun hexafluorophosphate ($[C_6MIM][PF_6]$) was purchased from Shanghai Chengjie Chemical Reagent Co. Ltd. Formic acid was of analytical-reagent grade and purchased from Beijing Chemical Factory (Beijing, China). Sodium chloride of analytical grade was purchased from Beijing Chemical Factory (Beijing, China). Anhydrous sodium sulfate of analytical grade was purchased from Tianjin Fengchuan Chemical Factory (Tianjin, China). Chromatographic grade acetonitrile was purchased from Fisher Scientific Company (UK) and pure water was obtained with a Milli-Q water purification system (Millipore Co., USA). Heparin saline（12500u Heparin：1000ml 0.9% saline； Hebei Changshan Biochemical Pharmaceutical Co. Ltd., Shijiazhuang, China), gavage needle, and sulfamethoxazole Tables(Shanghai New Asia Pharmaceutical Co. Ltd., Shanghai, China) were used.

2.2. Instruments

The 1100 series liquid chromatograph (Agilent Technologies Inc., USA) equipped with UV detector and quaternary gradient pump was used. Zorbax Eclipse Plus-C_{18} column (150mm×4.6mm, 3.5 μm,162 Agilent, USA) and a C_{18} guard column (7.5 mm× 2.1 mm I.D., 5 μm) were used. The KQ-100DE ultrasonic cleaner was purchased from Kunshan Ultrasonic Instrument Co., Ltd. (Kunshan, China). The frequency and output power of the ultrasonic cleaner are 40 kHz and 100 W, respectively. The SH-39 oscillator was purchased from Shanghai Zhenghui Instrument

Co., Ltd. (Shanghai, China).

2.3. Samples

In the study, five kinds of samples were used. The fresh human blood sample (Sample 1) was obtained from local hospital. Rabbit blood sample (Sample 2) was obtained from local animal experiment center. Pig (Sample 3), cow (Sample 4) and chicken (Sample 5) blood samples were obtained from local market. Except for the experiments mentioned in Section 3.3, which were performed with all five samples, all other results were obtained with sample 1. The spiked samples containing SAs were prepared by adding the working solutions into blood samples and stored at 4 ℃ for one week.

Five male Japan big-eared white rabbits (2.5-3.2 Kg, 5 months old) were provided by laboratory animal centre of Jilin University（License NO. SCXK（Ji）2009-0003） for the pharmacokinetic investigation. All animals were kept from birth under housing conditions (temperature: 22 ± 1°C; relative humidity, 60 ± 3 %; 12-hour light/darkness cycle; low noise disturbances), allowing free access to a standard diet in pellets with water. All experimental protocols were in accordance with the guidance suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China（The Ministry of Science and Technology of the Peoples Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30）.

2.4. HPLC-UV conditions

The mobile phase consists of acetonitrile containing 2.67 mmol L^{-1} formic acid (A) and 2.67 mmol L^{-1} formic acid aqueous solution (B). The gradient program is as follows: 0–10 min, 10–30 % A; 10–20 min, 30 -34 % A; 20–21 min, 34–35 %A;

21–24 min, 35 % A and 24–30 min, 35–10 % A. The flow rate of mobile phase was kept at 0.5 mL. min^{-1} and column temperature was kept at 40 ˚C. The injection volume of analytical solution was 10 μL. The monitoring wavelength was 270 nm.

2.5. Extraction procedure

The extraction procedure is shown in Fig. 2. 10 mL of blood sample was centrifuged at 5 ℃ for 10 min at 15,000 rpm. The upper serum was withdrawn into 10 mL syringe and then 200 μ L $[C_6MIM][PF_6]$ was withdrawn into the syringe. The syringe was immersed into the ultrasound bath for 2 min at 50℃, and then shaken for 6 min on oscillator. The resulting extract was cooled at 5 ℃ for 40 min. When the extract was cooled the hydrophobic ionic liquid was deposited in the inner surface of the syringe and upper surface of the plunger. When the plunger of the syringe was depressed, the serum phase was first pushed out and then the ionic liquid phase was pushed into 0.5 mL centrifuge tube. The anhydrous sodium sulfate was added in the centrifuge tube to remove the water. The resulting $[C_6MIM][PF_6]$ phase was filtered with 0.22 μ m PTFE filter membrane before analysis.

3 Results and discussion

3.1. Optimization of extraction conditions

In order to obtain high extraction efficiency, the effects of experimental parameters, including the volume of ionic liquid, salt concentration, pH value of system, extraction time, temperature and cooling time were investigated.

3.1.1. Salt concentration

Generally, the addition of salt in DLLME can cause a decrease in solubility of the analytes in water, and extraction efficiency can be improved. In order to investigate the effect of salt concentration on the performance of IL-DLLME a series of experiments were performed by adding NaCl (0–5%, w/v). As shown in Fig. 3, when the concentration of NaCl increases from 0 to 2%, the recoveries for analytes increase except for SDZ and the recovery of SDZ decreases slightly. However, when salt concentration is higher than 2%, the recoveries of the analytes decrease. Too high concentration of salt can cause the increase in viscosity of solution and the solubility of IL in water, which can result in the decrease of extraction efficiency. Finally, concentration of NaCl was selected as 2%.

3.1.2 Effect of pH of system

The solubility of **SAs** in water is related to the pH value, so the pH value has a significant effect on the extraction recoveries of the analytes. The effect of pH value of the sample solution in the range of 2.0-10.0 on the extraction recoveries was studied. The detailed information can be found in Fig. 4. The highest extraction recoveries are obtained when pH is 4.0. When the pH value of solution is higher than 7.0, the recoveries of SAs decrease significantly and the peak shape change. At low pH, SAs would be protonated, and are easily soluble in the aqueous phase. The log Pow (Pow, octanol-water partition coefficient) values of SAs are lower than 1.0, which means that SAs are easily soluble in water and not easily miscible with proteins and lipids. Therefore, when pH is low, SAs would easily distribute into the IL phase which is beneficial to the extraction of SAs. However, when pH is high, SAs are insoluble in water and easily miscible with proteins and liquids, which is not beneficial to the extraction of SAs. Therefore, the pH value of 4.0 was optimal.

3.1.3. Effect of extraction time

The extraction time includes ultrasonic time and oscillation time. Both ultrasonic and oscillation can effectively stimulate the transfer of the analytes from sample to the IL phase. The ultrasonic should be more effective than oscillation. However, long ultrasonic time can made the temperature of the system increase. So, both ultrasonic and oscillation were applied to the extraction of the analytes.

As shown in Fig. 5, the recoveries of the target analytes increase gradually with the increase of the ultrasonic time ranging from 1 to 2 min. The extraction time longer than 2 min would not affect the extraction efficiency. The extraction equilibrium can be achieved in short time and the phase-transfer of the target analytes is fast. Therefore, ultrasonic time of 2 min was selected.

The effect of oscillation time on the recoveries was evaluated. The detailed information can be found in Fig. 6. The recoveries of the target analytes increase gradually with the increase of the oscillation time ranging from 0 to 6 min. When oscillation time is longer than 6 min, the recoveries of the target analytes decrease. It may because long oscillation time can cause the loss of ionic liquid. Therefore, oscillation time of 6 min was selected.

3.1.4. Effect of extraction temperature

Temperature has a significant effect on solubility and mass transfer of the analytes. The increase of the temperature should be beneficial to the increase of the transfer rate of the analytes from the sample to the extraction solvent, which is beneficial to the increase of the recoveries of the analytes. However, excessively high temperature could result in the decomposition of the analytes and the increase of the solubility of IL, which is not beneficial to the increase of the recoveries of the analytes. The effect of extraction temperature on the recoveries was evaluated from 20 ℃ to 60 ℃. The recoveries of the analytes increase with the increase of temperature from 20 to 50 ℃,

and then remain unchanged up to 60 ℃. As shown in Fig. 7. The extraction temperature of 50 ℃ was chosen in this study.

3.1.5. Effect of cooling time

After the extraction was completed the syringe was cooled in ice water. The effect of the cooling time ranging from 0 to 60 min was examined. The detailed information can be found in Fig. 8. The recoveries of the target analytes decrease gradually with the increase of the cooling time from 0 to 20 min. When cooling time is longer than 20 min, the recoveries of the target analytes increase gradually with the increase of the cooling time. When the cooling time is longer than 40 min, the recoveries of the analytes slightly decrease. The temperature decreases with the increase of the cooling time. Both the temperature and time can affect the solubility of ionic liquid and the partition ratio of the analytes in the system. So the effect of cooling time is slightly complicated. Therefore, cooling time of 40 min was selected.

3.2. Analytical performances

SAs were determined under the above optimized conditions. Fig. 9 shows chromatograms of the standard solution and blood sample.

The working curves were constructed by plotting the peak areas measured versus the concentrations of analytes in the spiked samples. The linear regression equations and the correlation coefficients are listed in Table 1. The limit of detection (LOD) and quantification (LOQ) indicated in Table 1 are determined as the lowest concentrations yielding a signal-to-noise (S/N) ratio of 3 and 10, respectively.

Repeatability was evaluated by determining target analytes in spiked blood samples. The intra-day precision was obtained by analyzing the samples five times in one day. The inter-day precision was achieved by analyzing the samples once a day in five

consecutive days. The results are presented in Table 1 and indicate that the present method has good repeatability. Long-term stability of analytes in blood sample during sample storage was evaluated. The spiked samples were prepared according to the method mentioned in Section 2.3, kept in centrifuge tube and then stored for 1, 2, 3, 4, 6 and 8 weeks at 4 ℃, respectively. All experiments were performed in five replicates. The results are listed in Table 2. The recoveries and RSD values range from 87.0 to 115.3 % and from 0.7 to 7.2 %, respectively. It can be concluded that the SAs in the blood samples were stable for at least one month.

3.3. Analysis of samples

In order to evaluate the applicability of the present method, this method was applied to the determination of the residues of the SAs in blood samples. The results are listed in Table 3. It can be seen that the present method provides good recoveries (88.4-115.6%) and acceptable precision (≤ 7.3 %).

3.4. Pharmacokinetic investigation

Before the experiment, five male rabbits were starved for at least 12h, but water was given ad libitum. Oral doses (nominal 13 mg/kg body weight) of SMO was administered by syringe as a solution in distilled water (25–35ml). After dosing, rabbits were housed singly in stainless-steel cages， and 3mL blood samples were collected from ear vein in 2ml centrifuge tube after oral administration for 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12, 24, 48h, respectively. All the blood samples were stored at -20℃ until analyzed. The curve of SMO concentration in blood - time is shown in Fig. 10. The C_{max} of SMO is 8.2 μ g.mL^{-1} appearing about at 1h.

4. Conclusion

The in - syringe IL - DLLME was successfully applied to the extraction of the SAs from blood samples. The IL was used as extraction solvent. The extraction was carried out in a syringe. The separation of IL phase and serum phase was carried out by moving the plumger of the syringe. No centrifugation was required and the operation was simplified. The LODs and recoveries of the SAs were 1.58-12.30 ng. ml^{-1} and 88.4-115.6 %, respectivelly. The pharmacokinetic investigation was carried out and the curve of SMO concentration in blood – time was obtained. So it seems possible to extend this method to the extraction of SAs in other similar samples by varying the extraction conditions.

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343 ## References

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401 # Figure captions

402

403 **Fig. 1.** Chemical structures of SAs.

404 **Fig. 2.** Extraction procedure

405 **Fig. 3.** Effect of NaCl concentration on the recoveries of the analytes.

406 Sample amount, 10 mL; C_6[MIM]PF_6 volume, 200µl; Ultrasonication time 2 min; Oscillation time,

407 6 min; Cooling time, 40 min;

408 **Fig. 4.** Effect of pH concentration on the recoveries of the analytes.

409 Sample amount, 10 mL; C_6[MIM]PF_6 volume, 200µl; NaCl amount, 0.2 g; Ultrasonication time, 2

410 min; Oscillation time, 6 min; Cooling time, 40 min.

411 **Fig. 5.** Effect of ultrasonication time concentration on the recoveries of the analytes.

412 Sample amount, 10 mL; C_6[MIM]PF_6 volume, 200µl; NaCl amount, 0.2 g; Oscillation time, 6 min;

413 Cooling time, 40 min.

414 **Fig. 6.** Effect of oscillation time concentration on the recoveries of the analytes.

415 Sample amount, 10 mL; C_6[MIM]PF_6 volume, 200µl; NaCl amount, 0.2 g; Ultrasonication time, 2

416 min; Cooling time, 40 min.

417 **Fig. 7.** Effect of temperature concentration on the recoveries of the analytes.

418 Sample amount, 10 mL; C_6[MIM]PF_6 volume, 200µl; NaCl amount, 0.2 g; Ultrasonication time, 2

419 min; Oscillation time, 6 min; Cooling time, 40 min.

420 **Fig. 8.** Effect of cooling time concentration on the recoveries of the analytes.

421 Sample amount, 10 mL; C_6[MIM]PF_6 volume, 200 µ l; NaCl amount, 0.2 g; Ultrasonication time,

422 2 min; Oscillation time, 6 min.

423 **Fig. 9.** Chromatograms of standard solution (A) blood sample (B) and spiked blood sample (C)

424 1. SDZ; 2. SMZ; 3. STZ; 4. SCP; 5. SMX; 6. SSZ.

425 Sample amount, 10 mL; C_6[MIM]PF_6 volume, 200µl; NaCl amount, 0.2 g; Ultrasonication time, 2

426 min; Oscillation time, 6 min; Cooling time, 40 min. The concentration of analytes in standard

427 solution: SDZ, 532 ng. mL^{-1}; SMZ, 535 ng. mL^{-1}; STZ, 589 ng. mL^{-1}; SCP, 543 ng. mL^{-1}; SMX,

428 532 ng. mL^{-1}; SSZ, 570 ng. mL^{-1}.The concentration of the analytes in spiked sample: SDZ, 23.1

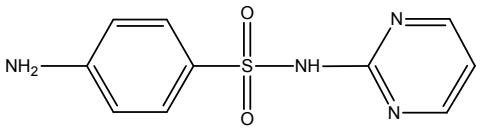
429 ng. mL^{-1}; SMZ, 23.3 ng. mL^{-1}; STZ, 25.6 ng. mL^{-1}; SCP, 23.6 ng. mL^{-1}; SMX, 23.1 ng. mL^{-1}; SSZ,

430 24.8 ng. mL^{-1}.

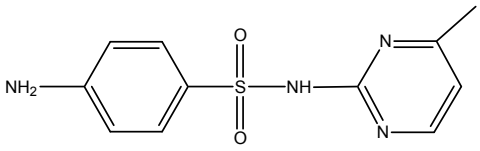
431 **Fig.10.** Curve of SMO concentration in plasma - time

432 Sample amount, 10 mL; $C_6[MIM]PF_6$ volume, 200μl; NaCl amount, 0.2 g; Ultrasonication time, 2

433 min; Oscillation time, 6 min; Cooling time, 40 min.



SDZ



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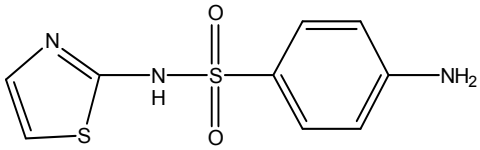

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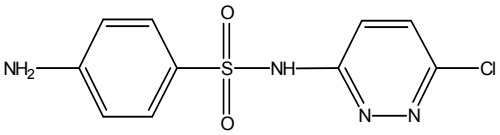
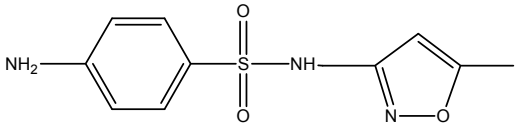
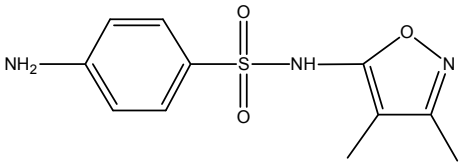


SCP



SMX



SSZ

Fig. 1.

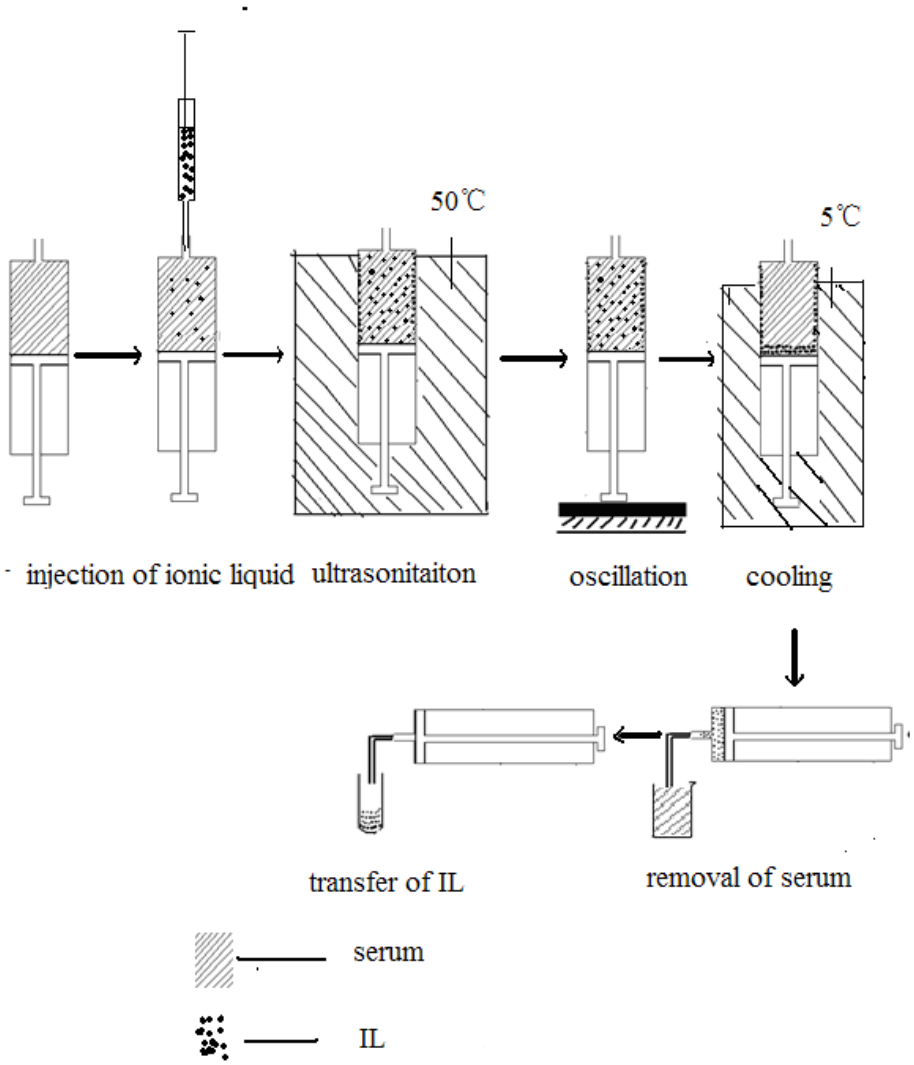


Fig. 2

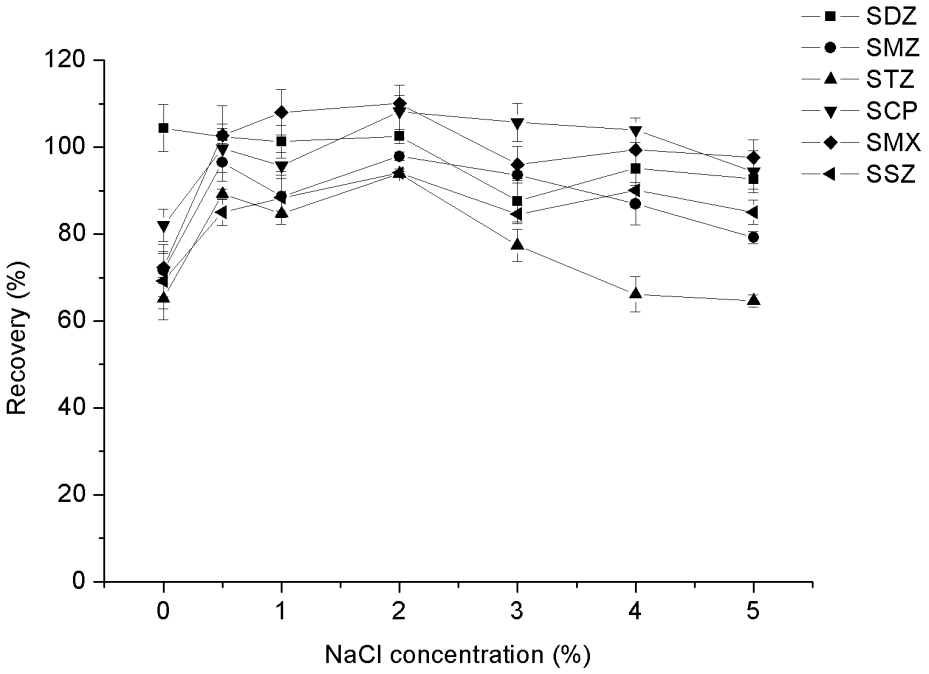


Fig. 3

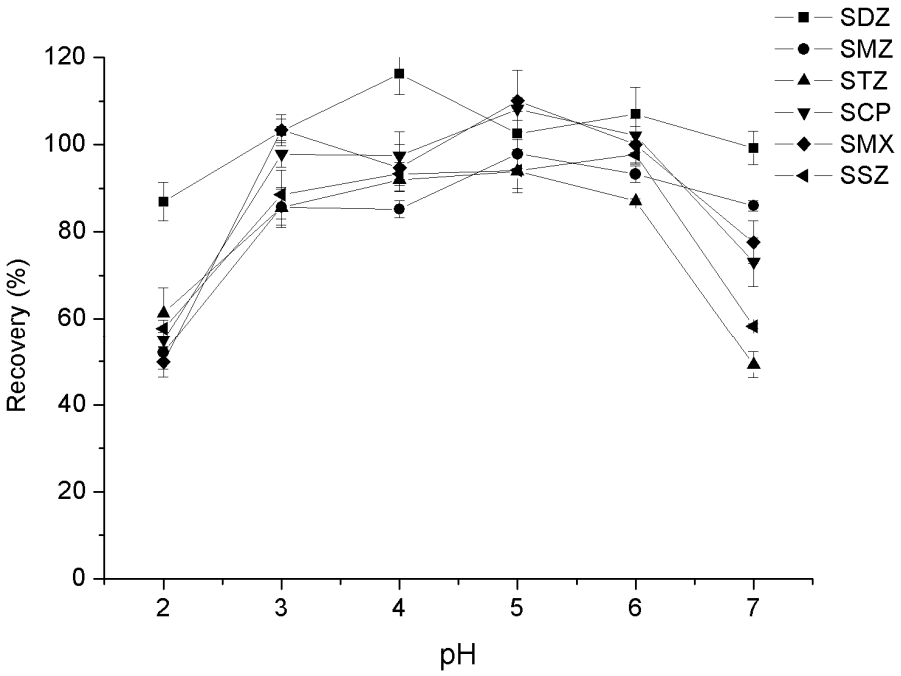
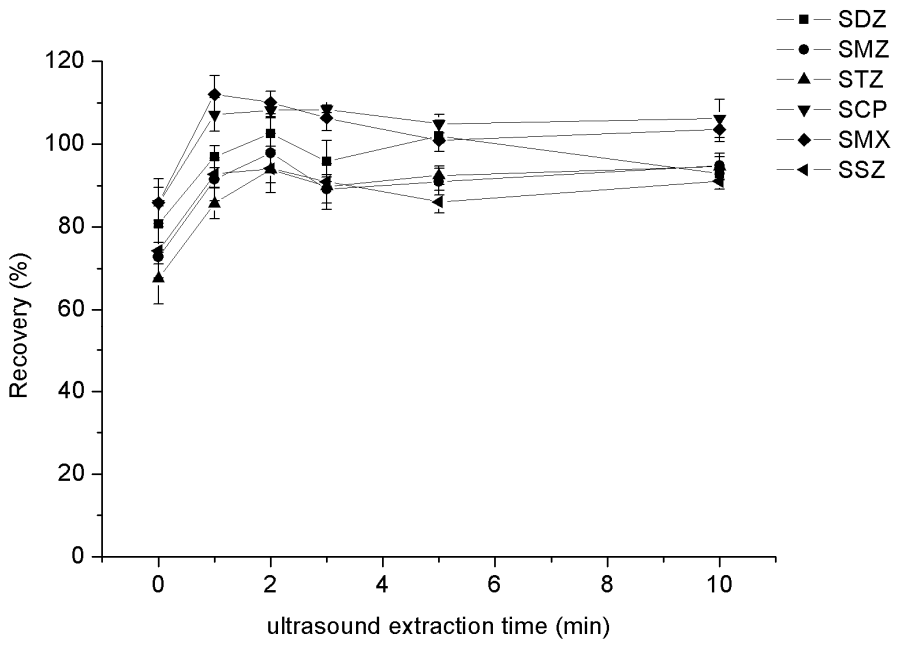


Fig. 4



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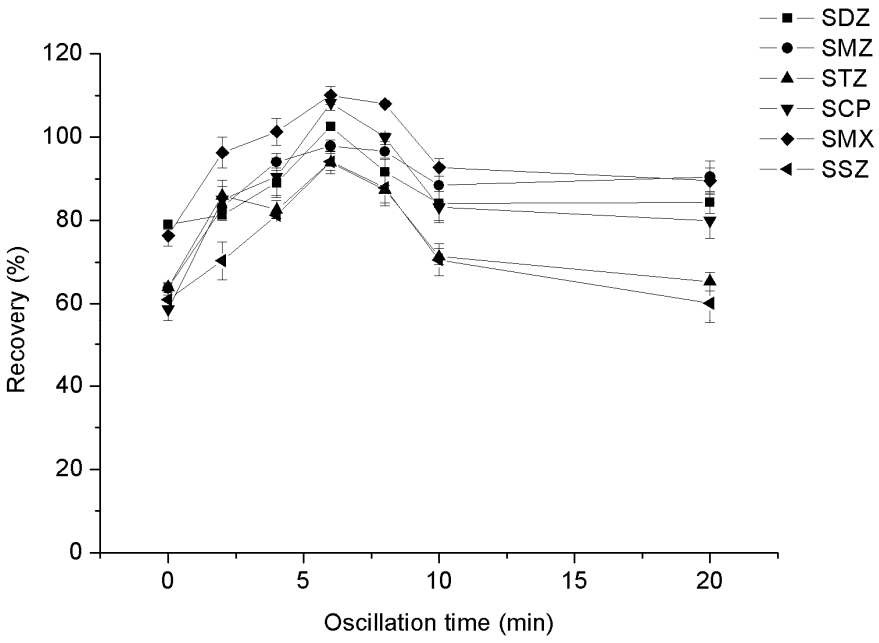

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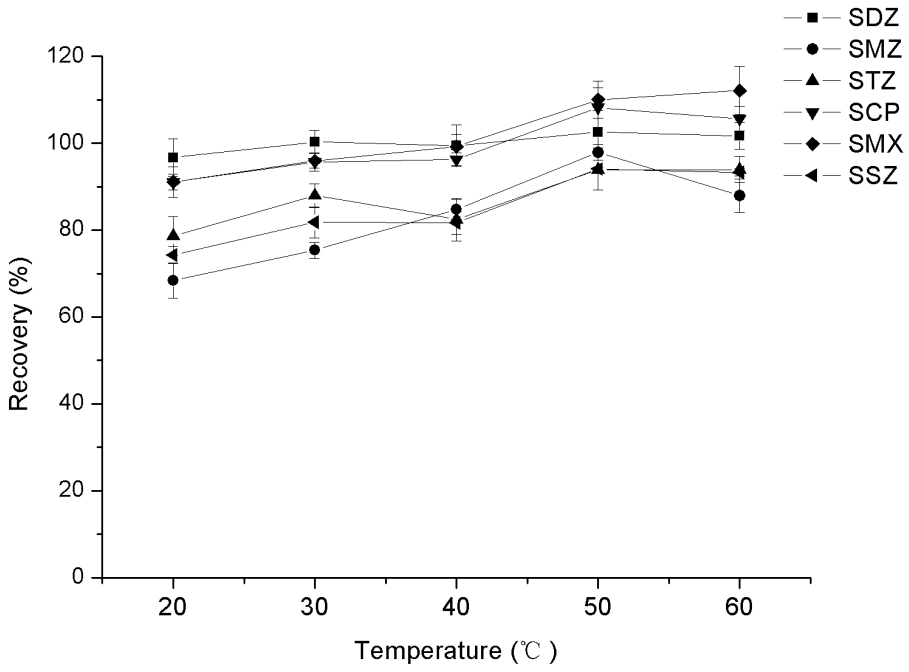

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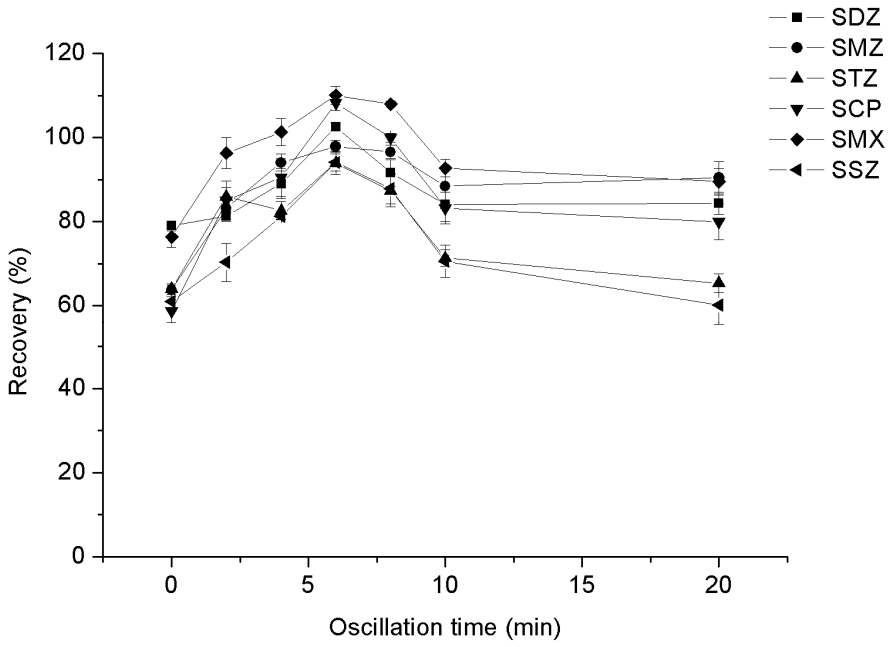
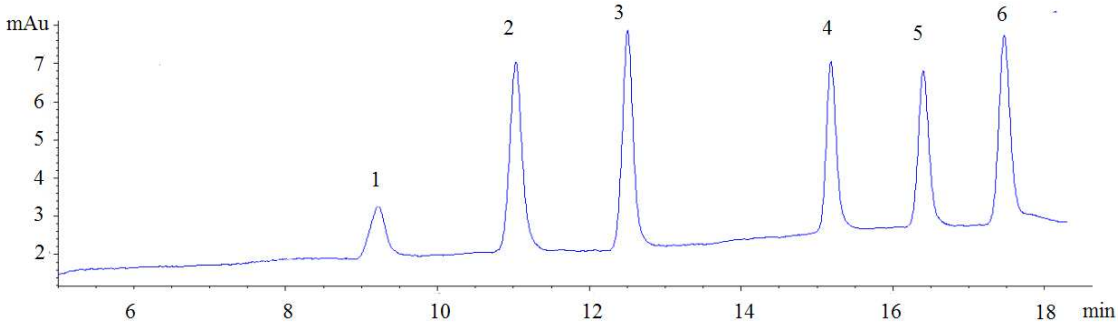


Fig. 8



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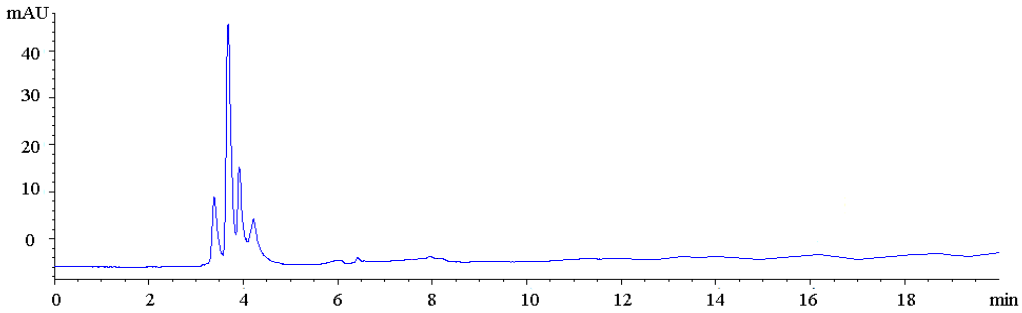

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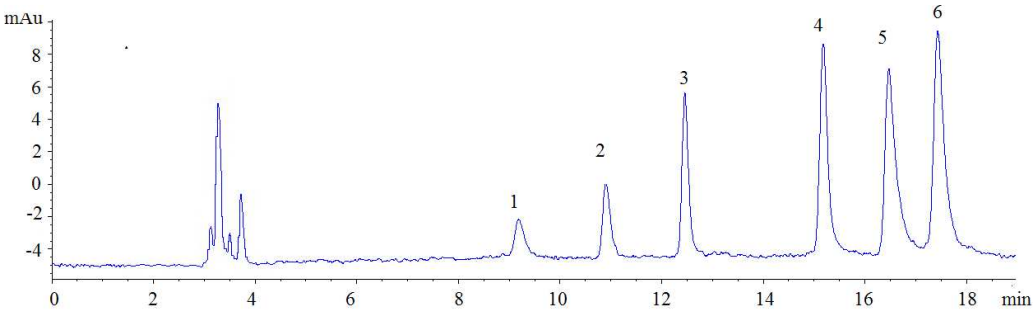

C

Fig. 9

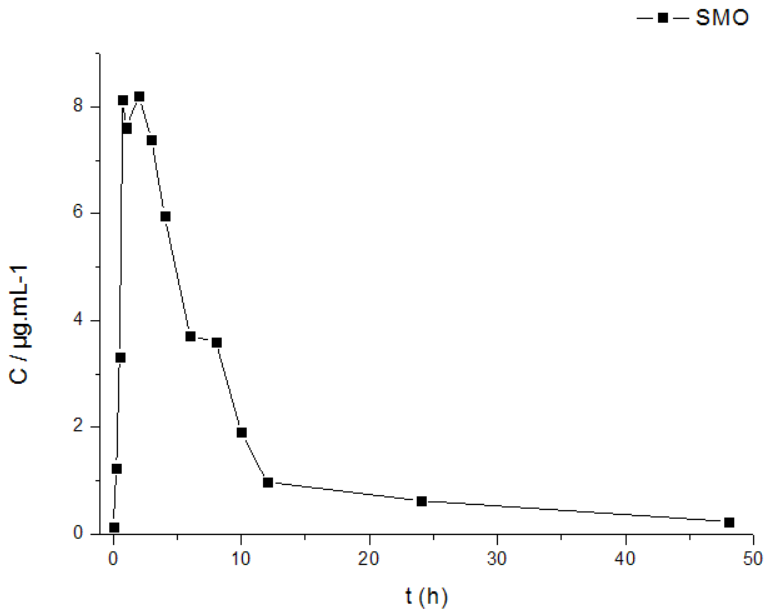

Fig. 10

Table 1

Analytical performances

Compound	Corrlation coefficient (r)	Regression equation (n=5)	Linear range(ng·mL^{-1})	LOD (ng·mL^{-1})	LOQ (ng·mL^{1})	Intra day precision (RSD, %, n=5)	Inter day precision
SDZ	A=(-2.89±4.197^{a})+(0.70±0.025^{b})c	0.9980	43.8-442.6	12.30	41.0	2.7	2.9
SMZ	A=(-0.23±0.555^{a})+(1.41±0.007^{b})c	0.9999	8.4-385.8	2.22	7.4	2.2	3.8
STZ	A=(3.11±2.017^{a})+(2.60±0.024^{b})c	0.9998	13.9-458.2	3.19	10.6	2.4	4.7
SCP	A=(1.14±1.681^{a})+(3.01±0.020^{b})c	0.9999	8.1-377.3	1.67	5.6	1.8	5.4
SMX	A=(-1.16±1.804^{a})+(3.02±0.022^{b})c	0.9999	14.4-417.0	2.93	9.8	3.5	4
SSZ	A=(-0.77±6.619^{a})+(4.97±0.080^{b})c	0.9996	9.8-398.5	1.58	5.3	2.0	4.9

^{a,b} Standard deviation of slope and intercept.

Table 2

The recoveries of the analytes in spiked sample 1

		STZ		SMZ		STZ		SCP		SMX		SSZ	
spiked	Stored time	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD
(μg.L-1)	(weeks)	(%)	(% n=5)	(%)	(% n=5)	(%)	(% n=5)	(%)	(% n=5)	(%)	(% n=5)	(%)	(% n=5)
50	1	100.2	3.3	101.5	4.1	92.1	2.5	109.9	5	112.7	6.1	93.6	4.2
	2	98.3	1.8	92.8	2.2	96.8	5.2	106.2	2.1	105	3	96.8	2.9
	3	106.2	5.6	99	6.5	93.9	3.7	107	3.9	103.7	2.7	90.4	2.8
	4	97.2	0.9	102.6	1.9	89.5	3.9	103.4	3.6	110.4	3.8	93.9	3.8
	6	102.1	5.2	95.4	5	94.2	4.6	101.2	1.7	115.3	3.3	92.1	5
	8	100.3	5.9	97.4	3.7	88	1.9	110.6	5.6	101.1	4.6	95	3.5
150	1	95.6	3.6	100	4.2	89.2	3.2	106.5	1.4	113.8	6.5	89.7	2.3
	2	100.9	3.8	98.2	2.5	90	4.5	112.1	4.5	101	4.5	98.3	1.6
	3	105	4.1	95.6	1.4	92.4	3.3	104.5	6	107.5	3.1	91.7	2.2
	4	97.5	1.6	93.7	3	91.7	0.7	108.6	1.4	106.3	4	94	4
	6	99.4	2.7	95.5	4.1	96.9	7.1	99.9	3.1	113.4	7.2	88.9	4.6
	8	98.7	4.2	104.6	6.2	87	2.9	104	2.8	111.1	5.3	93.5	1.3

Table3

Analytical results of blood samples (n=5)

Add (μg.Kg-1)	Added (μg L-1)	STZ		SMZ		STZ		SCP		SMX		SSZ	
		Recovery (%)	RSD (% n=5)	Recovery (%)	RSD (% n=5)	Recovery (%)	RSD (% n=5)	Recovery (%)	RSD (% n=5)	Recovery (%)	RSD (% n=5)	Recovery (%)	RSD (% n=5)
Sample 1	50	104.3	4.2	98.7	1.7	92.7	2.8	106.4	3.1	105.1	1.7	95.1	3.8
	150	99.1	3.7	101.5	4.8	107.2	4.7	113.7	3.7	111.5	2.5	98	3.6
Sample 2	50	110.6	4.9	95	3.2	94.6	5.2	105	5.8	103.2	2.6	93.7	2.9
	150	107.3	7.1	95.4	5	102.1	5	98.9	1.6	100.9	3.9	97.5	1.7
Sample 3	50	97.5	5.3	100.2	3	101.3	3.9	97.5	3.5	105.4	4.1	102.5	5.6
	150	105.8	2.9	104.8	2.2	98	1.9	108.2	4.8	115.6	7.3	99.7	2.8
Sample 4	50	96.9	1.6	96.3	4.1	94.4	3.8	99.6	4.4	100.2	4.4	91.9	4.5
	150	93.8	5	97.9	3.7	96.8	3.9	103.5	2.8	95.5	2.4	88.4	5.3
Sample 5	50	106.39	4.4	103.6	3.6	91.4	2.3	109	5.3	98.7	3.9	97.8	3
	150	101	0.9	100.4	4.9	101.9	6.8	105.8	2.6	104.3	3.1	90.3	4.9