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Simultaneous determination of four synthesized metabolites of mequindox in urine samples using ultrasound-assisted dispersive liquid–liquid microextraction combined with high-performance liquid chromatography

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ABSTRACT

A novel pretreatment method termed ultrasound-assisted dispersive liquid–liquid microextraction (UADLLME) coupled with high-performance liquid chromatography-ultraviolet detector (HPLC-UV) was applied for the detection of four synthesized metabolites of mequindox in pig urine samples. A total volume of 200 μL of methanol (dispersant) and 60 μL of 1,1,2,2-tetrachloroethane (extract) were injected into 5.0 mL of urine sample and then emulsified by ultrasound treatment for 4 min to form a cloudy solution. The effect of several factors on the recovery of each metabolite was investigated by a fitting derivation method for the first time. Under optimum conditions, the method yields a linear calibration curve in the concentration range from 0.5 to 500 μg/L and a limit of detection (LOD) of 0.16–0.28 μg/L for target analytes. The recoveries ranged from 72.0% to 91.3% with a relative standard deviation (RSD) of less than 5.2%. The enrichment factors for the four compounds ranged from 75 to 95. Two pig urine samples were successfully analyzed using the proposed method.

1. Introduction

Since the discovery of quinoxaline, a number of structural modifications to the quinoxaline nucleus have been made to increase the antimicrobial activity and to enhance the pharmacokinetic performance of these compounds. Many mono- and di-N-oxides and 2-oxo derivatives of this heterocyclic system were generated, and their biological activities were reported [1]. In the last three decades, quinoxaline-1,4-dioxides (QdNOs) derivatives have been regarded as a versatile range of pharmacological active drugs, and quindoxin, carbadox and olaquindox are all known members of this class of compounds [2]. These QdNOs derivatives are widely used as medicinal feed additives for ameliorating the intestinal microflora, improving protein utilization, and increasing protein synthesis *in vivo* [3]. Unfortunately, problems pertaining to the toxicity of these drugs have their application [4,5], and the European Union (EU) banned the product license of carbadox and olaquindox in 1999 [6]. Mequindox (2-methyl-3-acetylquinoxaline-1,4-dioxide; $C_{11}H_{10}N_2O_3$; MEQ) is a novel synthetic QdNOs derivative that was developed by the Lanzhou Institute of Animal Husbandry and Veterinary Drugs, Chinese Academy of Agriculture Sciences. MEQ has been applied to the treatment of diseased piglets, including those with white diarrhea and swine dysentery [7]. Although MEQ was considered to have better growth-enhancing activity and to be safer than carbadox and olaquindox when administered to animals [2], very little information was available about the cytotoxicity and genotoxicity, and the metabolomics of mequindox are quite poorly understood.

To the best of our knowledge, the majority of pharmacokinetic studies concerning veterinary chemicals have focused on their metabolites. Certain metabolites of quinoxaline-1,4-dioxide derivatives, especially their desoxy and reduction compounds, are suspected carcinogens and mutagens, which cause severe side effects and triggered safety concerns [8,9]. According to recent research, several metabolites of MEQ were found exist in rat, chicken and pig liver microsomes [10]. The research also indicated that mequindox was mainly metabolized to 1,4-bisdesoxy-mequindox (1,4-BDM), 1-desoxy-maquindox (1-DM), 4-desoxy-maquindox (4-DM) and 1-(3-methyl-quinoxalin-2-yl)-ethanol (MEQE). Together, these four main metabolites have been shown to account for more than 60% of the relative percentage of MEQ metabolites in liver microsomal incubations of rats and chickens [10]. This indicated that these metabolites could be promising residual markers of MEQ. For practical applications and concerns over food-safety, it is necessary to develop sensitive and accurate analytical methods for the quantification of these metabolites.

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While high-performance liquid chromatography (HPLC) with UV detection is the most widely applied method to detect residues of veterinary drugs, only a few methods have been developed for QdNOs derivatives [11,12]. For example, a HPLC method for the simultaneous quantitative determination of five QdNOs derivatives (carbadox, olaquindox, cyadox, mequindox, quinocetone) in feeds has been described by Wu et al. Another paper described a HPLC-UV-based method for the determination of levels of cyadox and its metabolites in plasma and tissues of chicken. However, there is no published reported that describe a method for the simultaneous measurement of mequindox metabolites in biological fluid samples using HPLC.

Sample preparation is one of the most crucial steps in the whole analytical process. It is also the bottleneck for obtaining accurate and sensitive results, especially in the detection of trace analytes within complex matrices [13]. In general, this step consists of extraction and preconcentration of target compounds from a sample matrix. The most popular pretreatment methods are liquid–liquid extraction (LLE) and solid phase extraction (SPE). Nevertheless, these conventional extraction methods are laborious, time-consuming and require large volumes of samples and toxic organic solvents. Recently, much attention has been paid to the development of miniaturized, efficient and economical sample preparation methods; thus, several novel microextraction techniques are being developed to reduce the time required for analysis, to increase the sample throughput and to improve the quality and the sensitivity of the analytical methods [14]. In 2006, Rezaee and coworkers developed an emerging technique named dispersive liquid–liquid microextraction (DLLME) [15]. DLLME is generally carried out using a ternary component solvent system in which extraction and disperser solvents are rapidly injected into the aqueous sample, resulting in a cloudy solution similar to cloud point extraction. DLLME avoids many of the shortcomings of conventional methods previously mentioned and has been successfully applied for the pre-concentration of organic compounds in environmental and biological fluid samples [16–18].

In this work, 1,4-BDM, 1-DM, 4-DM and MEQE were synthesized. In addition, a novel method for the detection and quantification of these four main metabolites of mequindox, using ultrasound-assisted dispersive liquid–liquid microextraction (UADLLME) and high-performance liquid chromatography-UV photodiode-array detection, is presented. The application of ultrasonic radiation in DLLME methods accelerates the dispersion process and increases the extraction ability. Throughout the extraction procedures, factors influencing the extraction efficiency and detection were evaluated in detail. The objective of this work was to contribute to the residue monitoring of MEQ and to facilitate further pharmacokinetic and residue studies of similar QdNOs veterinary drugs. The presented methodology was successfully applied to simultaneously detect these four metabolites in samples of pig urine for the first time.

2. Experimental

2.1. Chemicals

2.1.1. Synthesis of 1-DM, 4-DM, 1,4-BDM and MEQE

The syntheses of 1-DM (purity ≥ 98%), 4-DM (purity ≥ 99%) and 1,4-BDM (purity ≥ 99%) were carried out following the protocol described in our previous work [19,20]. MEQE was prepared from 1,4-BDM. KBH_4 (aq, 0.3 mol/L) was added dropwise to a solution of 1,4-BDM (2.59 g, 0.0147 mol) in anhydrous alcohol at a temperature of about 30 °C. Reactions were monitored by TLC (thin-layer chromatography, using ethyl acetate/petroleum ether mixtures, 1:1) using precoated silica gel aluminum plates containing a fluorescent indicator. Reactions should be stopped when there was only one point visible by TLC. Most of the solvent was subsequently removed using a rotary evaporator. Water (30 ml) was added, and the mixture was then washed three times with chloroform (30 ml at a time). The organic phases were combined and dried with anhydrous sodium sulfate. Crude MEQE was then obtained by removing the solvent on a rotary evaporator. The crude product was dissolved in hexane (80 ml, 60 °C) and kept at 4 °C for 12 h. The resulting white crystals were then separated from the mixture. The recrystallization was repeated three times to give pure MEQE (1.93 g, 0.0111 mol, 75.5%, purity ≥ 99%). For MEQE: m.p. 77.8–78.7 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.56 (3, d, J = 6.5 Hz, 2-CH3), 2.76 (3, s, 3-CH3), 4.74 (1, d, J = 7.3, –OH), 5.18 (1, m, 2-CH), 7.73 (2, m, H-6, 7), 8.03 (2, m, H-5, 8); ^{13}C NMR (CDCl3, 75 MHz) δ 21.88, 23.43, 66.61, 128.29, 128.37, 129.27, 129.56, 139.52, 141.61, 151.07, 1596.91; IR (KBr) ν: 762, 888, 1038, 1271, 1440, 1489 cm^{-1}. The structures of these four compounds and the main metabolic pathway of MEQ in pigs according to Liu et al. [10] are displayed in Fig. 1.

2.1.2. Reagents

Maquindox (98% purity) was provided by the College of Veterinary Medicine, Huazhong Agricultural University (Wuhan, China). Other chemicals used in the synthesis were chloroform ($CHCl_3$), tetrachloromethane (CCl_4), 1,1,2,2-tetrachloroethane ($C_2H_2Cl_4$), sodium chloride (analytical grade), and trifluoroacetic acid (TFA), which were purchased from the Beijing Chemical Reagent Company. Dispersive solvents, including methanol, acetone, and acetonitrile (HPLC-grade), were purchased from Dikma Ltd. (China). Double distilled water was used for the preparation of aqueous solutions.

2.2. Instrumentation

Chromatographic analysis was carried out on an Agilent 1200 HPLC system equipped with a VWD detector system (California, USA). A high-pressure injection valve fitted with a 20 μL loop was used for the sample injection. The separation of the analytes was carried out on an Agilent Eclipse Plus C18 column (5 μm, 4.6 mm × 250 mm). A methanol–acetonitrile–water (26:26:48, v/v) mixture with 0.1% TFA was used as the mobile phase at a flow rate of 1 mL/min. The injection volume was 10 μL, and the detection wavelength was 320 nm. Analytes were weighed with a Mettler-Toledo AL104 electronic balance (Shanghai, China). Centrifugation was performed in a 52a centrifuge from the Baiyang Centrifuge Factory (Baoding, China) at 3500 rpm. The samples were ultrasonically irradiated in a water bath at 150 W and 40 kHz using an ultrasonic instrument (KQ3200DE; Kunshan Ultrasonic Instrument Co. Ltd., Kunshan, Chian). All the glassware used throughout this work was washed with deionized water and acetone and then dried at room temperature.

2.3. Sample preparation

Two urine samples were collected from female changbai pigs (purchased from Zhoukou Nursery), which were allowed free access to standard food and tap water for least 1 week before the experiment. The obtained samples were stored at −20 °C until the time of analysis. Prior to analysis, the frozen urine samples were thawed at room temperature and centrifuged for 10 min at 3500 rpm. The supernatants were transferred to another tube and filtered through a 0.22 μm filter membrane (Agla, USA). The resulting solutions were then subjected to the UADLLME process.

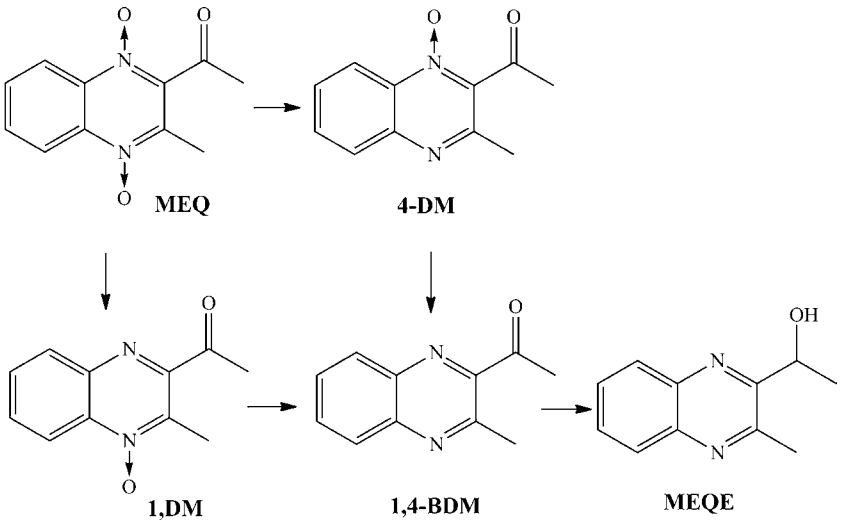

Fig. 1. The main metabolic pathway of MEQ in pig and structures of 1-DM, 4-DM, 1,4-BDM and MEQE.

2.4. Standard solutions and calibration curves

Stock standard solutions of the four metabolites were prepared by dissolving each (500 mg/L) in acetonitrile. Working solutions were prepared by diluting the stock solutions with acetonitrile to obtain different concentrations ranging from 0.01 to 10 mg/L. Stock standard solutions were stable for 6 months, and each working standard solution was stable for no longer than 3 months when stored in the dark at 4 °C. It had been recognized that QdNOs derivatives were sensitive to light, and Macintosh et al. found that the desoxy compounds were more sensitive to light than the parent compounds [21,22]. Therefore, it was necessary to protect the samples from strong light during preparation. Quantitative analysis was performed using an external standard. A series of urine samples prepared by diluting appropriate aliquots of the stock solution were subjected to the optimal UADLLME procedure. The urine samples were used for validation of method and application in real samples. The calibration curve for each metabolite was obtained by simple linear regression using a plot of concentration versus peak area, and the concentration of analyte in each sample was calculated using the resulting calibration curve.

2.5. UADLLME procedure

A homogeneous sample solution (5 mL) was spiked at 100 μg/L and was placed in a 15 mL screw-cap polytetrafluoroethylene tube with a conical bottom containing 0.1 g NaCl. An appropriate mixture of an extractant (60 μL 1,1,2,2-tetrachloroethane dissolved in 200 μL methanol) was rapidly injected into the aqueous sample. A cloudy solution that consisted of fine droplets of $C_2H_2Cl_4$ dispersed into the aqueous sample was formed, and the analytes were extracted into the fine droplets. The conical tube was subjected to ultrasonic treatment for 4 min to ensure complete extraction. The mixture was centrifuged at 3500 rpm for 10 min. Subsequently, the aqueous samples and urine samples were processed differently. In the case of aqueous samples, a small droplet of 1,1,2,2-tetrachloroethane was sedimented to the bottom of the conical test tube. For the urine samples, a white lipidic solid was sedimented to the bottom of the conical test tube, probably due to the co-sedimentation of the matrices (such as carbamide and uric acid) in urine [23]. Hence, for aqueous samples, the upper aqueous phase was carefully removed with a syringe and an appropriate volume of sedimented phase was injected into the HPLC system for analysis. In the case of urine samples, the aqueous solution was first slowly discarded and the resulting droplet and lipidic solid were dissolved in acetonitrile (50 μL) before being filtered through a 0.22 μm membrane to remove the white floccule in the extract of urine. Finally, 10 μL of the extract was withdrawn and injected into the HPLC system for analysis.

3. Results and discussion

3.1. Optimization of UADLLME

To obtain the maximal extraction efficiency, some experimental parameters that would influence the extraction performance were investigated in detail. The following equations were used to calculate the enrichment factor and recovery.

$$\mathrm{EF} = \frac{C_{\mathrm{sed}}}{C_0} \tag{1}$$

where EF, C_{sed}, and C_0 are the enrichment factor, the analyte concentration in the sediment obtained from the calibration graph of direct injection of standard solution, and the initial concentration of analyte within the sample, respectively.

$$R\% = \frac{C_{\mathrm{sed}} V_{\mathrm{sed}}}{C_0 V_{\mathrm{aq}}} \times 100 = \mathrm{EF} \times \frac{V_{\mathrm{sed}}}{V_{\mathrm{aq}}} \times 100 \tag{2}$$

where $R\%$, V_{sed}, V_{aq} are the extraction recovery, the volume of the sediment phase, and the volume of the sample, respectively. A series of experiments were designed to optimize these parameters and to evaluate the extraction efficiency using the calculated values for EF and $R\%$.

3.1.1. Selection of the extraction solvent

The selection of an appropriate extraction solvent is important for DLLME methodology. According to the principles of DLLME, the extraction solvent should meet the following requirements: low solubility in water, higher density than water, superior extraction of the analytes and good chromatographic behavior [24]. Based on these considerations, three solvents, $CHCl_3$ (density 1.48 g/mL), CCl_4 (density 1.59 g/mL), and $C_2H_2Cl_4$ (density 1.60 g/mL), were selected. To evaluate the effect of volume of extraction solvent on extraction efficiency, all the other parameters except for volume of ionic liquid were kept constant. Based on the variation in density, the volume of sediment for each extraction solvent was different. The volume of sediment after UADLLME with 80 μL of $CHCl_3$, CCl_4, and $C_2H_2Cl_4$ were 35 μL, 50 μL and 62 μL, respectively. Fig. 2 shows

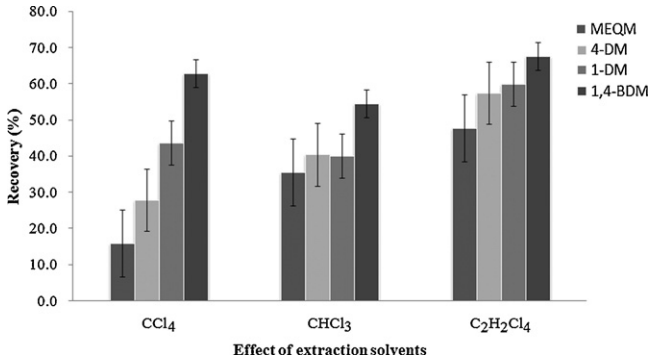

Fig. 2. Effect of different extraction solvent on the efficiency of UADLLME. Extraction conditions: concentration of analytes, 100 μg/L; sample volume, 5.0 mL; extraction solvent volume, 80 μL; disperser solvent, methanol; dispersive solvent volume, 200 μL; ultrasonic time, 1 min; centrifugation time, 5 min; without salt addition; room temperature.

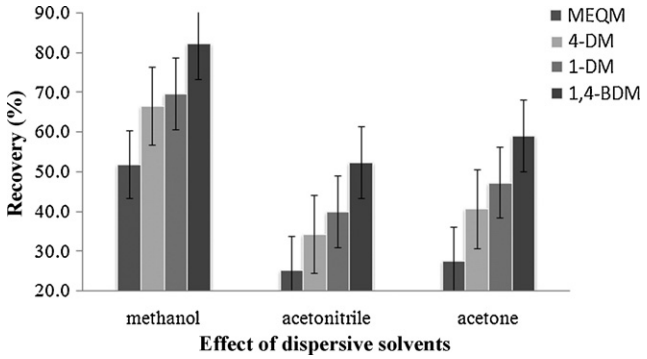

Fig. 4. Effect of dispersive solvents on the efficiency of UADLLME. Extraction conditions: concentration of analytes, 100 μg/L; sample volume, 5.0 mL; extraction solvent, 1,1,2,2-tetrachloroethane; volume of extraction solvent, 60 μL; dispersive solvent volume, 200 μL; ultrasonic time, 1 min; centrifugation time, 5 min; without salt addition; room temperature.

the effect of extraction solvent on the extraction recoveries. The largest recoveries were obtained using $C_2H_2Cl_4$ as the extraction solvent for all the analytes studied. Therefore, $C_2H_2Cl_4$ was selected for use in this study.

3.1.2. Effect of extraction solvent volume

The influence of the extraction solvent volume on the extraction performance was also investigated; 200 μL of dispersive solution (methanol) containing different volumes of 1,1,2,2-tetrachloroethane in the range of 40–90 μL were subjected to the same UADLLME procedure. It was observed that the sediment phase volumes increased as the volume of extraction solvent increased from 40 to 90 μL. As shown in Fig. 3, as the amount of 1,1,2,2-tetrachloroethane is increased, the extraction recoveries for the four analytes initially increased, reached a peak value at 60 μL and then slightly decreased. This is probably because large extraction solvent droplets that would rapidly settle at the bottom of the tube caused low extraction efficiencies. In contrast, the enrichment factors showed a continuous decreasing trend from 72–140-fold to 30–47-fold, which is seemingly due to the smaller sediment phase containing a higher analyte concentration after the UADLLME procedure. Although the EFs were decreased, the first derivatives of approximately 60 μL displayed smaller curves than the others. Consequently, 60 μL was used as the optimum volume of extraction solvent because the highest recoveries were obtained and the EFs were acceptable.

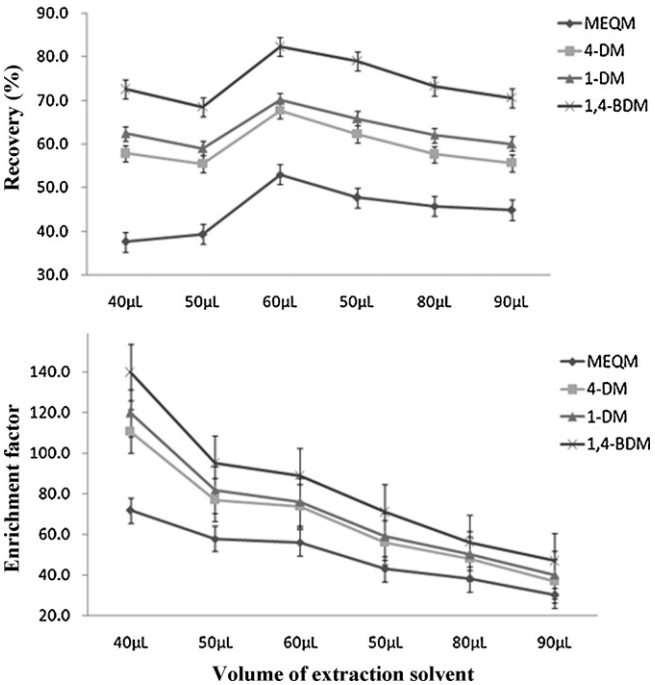

Fig. 3. Effect of volume of 1,1,2,2-tetrachloroethane as extraction solvent on the efficiency of UADLLME. Extraction conditions: concentration of analytes, 100 μg/L; sample volume, 5.0 mL; extraction solvent, 1,1,2,2-tetrachloroethane; disperser solvent, methanol; dispersive solvent volume, 200 μL; ultrasonic time, 1 min; centrifugation time, 5 min; without salt addition; room temperature.

3.1.3. Selection of dispersive solvent

The miscibility of the dispersive solvent in both the extraction and aqueous phase is an important factor affecting extraction performance in DLLME. Thus, several dispersive solvents were studied, including methanol, acetonitrile, and acetone. With 60 μL 1,1,2,2-tetrachloroethane as an extraction solvent, a normal cloudy phase system was formed with all three disperser solvents. Methanol is the most miscible with both pyrethroid and water, and the dispersion phenomenon was ranked in the following order: methanol > acetone > acetonitrile. The recoveries results, illustrated in Fig. 4, correlate with the dispersive properties of each solvent and indicated that methanol exhibited the highest extraction efficiency. In addition, the solvent peak of methanol does not interfere with the analyte peaks. Therefore, methanol was chosen as the disperser solvent in the present studies.

3.1.4. Effect of dispersive solvent volume

The volume of the disperser solvent is one of the key parameters in DLLME procedures. This parameter directly affects the formation of the cloudy phase solution and the degree of dispersion of the extraction solvent in the aqueous phase, thus affecting the extraction recoveries. To study this effect, the volume of methanol was varied between 200 and 1000 μL. Tiny droplet formation and cloudy state were stable when using all the selected volumes of dispersive solvent. The extraction results are shown in Fig. 5. An increase in the volume of the disperser solvent resulted in a decrease in the quantity of 1,1,2,2-tetrachloroethane in the sedimented phase. A possible explanation may be that, at high volumes, the solubility of analytes in water increases, which causes the extractor solvent to be hard to separate from the disperser and the aqueous solution. Moreover, the extraction efficiency decreased by increasing the methanol from 200 to 1000 μL. Hence, 200 μL of methanol was chosen as the optimum volume of dispersive solvent. The small quantity of organic solvent used during UADLLME is one of the most remarkable advantages of this technique.

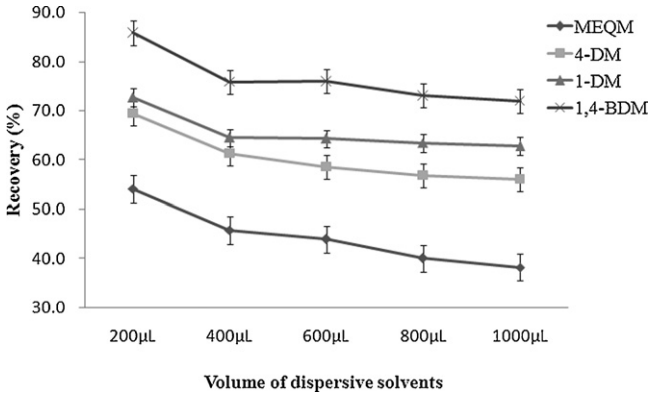

Fig. 5. Effect of volume of dispersive solvents on the efficiency of UADLLME. Extraction conditions: concentration of analytes, 100 μg/L; sample volume, 5.0 mL; extraction solvent, 1,1,2,2-tetrachloroethane; volume of extraction solvent, 60 μL; dispersive solvent, methanol; ultrasonic time, 1 min; centrifugation time, 5 min; without salt addition; room temperature.

3.1.5. Influence of ionic strength

To evaluate the possibility of any observable salting-out effect, experiments were performed in which different amounts of NaCl (0–8%, w/v) were added, while all other experimental conditions were kept constant. Fig. 6 displays the effect of adding NaCl on the recoveries of the four compounds. As the level of NaCl added was increased from 0 to 2%, the recoveries for all analytes increased accordingly. However, continuing to increase the salt concentration resulted in decreased extraction efficiency. Based on these results, 2% (m/v) NaCl was chosen as the optimal salt concentration in the UADLLME procedure.

3.1.6. Effect of ultrasonic time

Ultrasound treatment is a key factor in UADLLME, which directly influences the levels of dispersion. Therefore, the ultrasound-assisted processing time was varied from 1 to 5 min to evaluate its effect. As discussed in the research of Zhou et al., sufficient treatment time accelerates the formation of a fine dispersive mixture and results in higher recoveries. However, the extension of ultrasonic treatment time can also result in the loss of volatile analytes and extractants due to heat generation [25]. As illustrated in Fig. 7, it was found that all extraction performances reached the highest level (60.1%, 74.6%, 79.3% and 84.7% for MEQE, 4-DM, 1-DM and 1,4-BDM, respectively) when using an ultrasonic treatment time of 4 min. When the ultrasonic treatment time was longer or shorter than 4 min, the percentage recovery also decreased; thus, a 4 min treatment time was chosen for the following study.

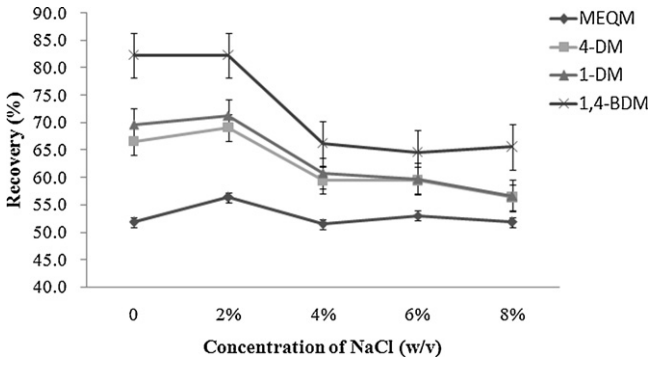

Fig. 6. Effect of concentration of NaCl on the efficiency of UADLLME. Extraction conditions: concentration of analytes, 100 μg/L; sample volume, 5.0 mL; extraction solvent, 1,1,2,2-tetrachloroethane; volume of extraction solvent, 60 μL; dispersive solvent, methanol; dispersive solvent volume, 200 μL; ultrasonic time, 1 min; centrifugation time, 5 min; without salt addition; room temperature.

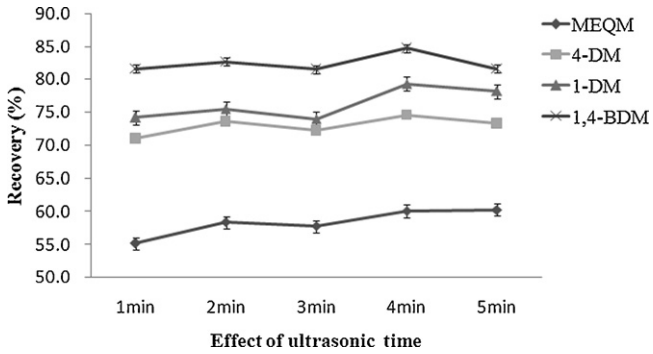

Fig. 7. Effect of ultrasonic time on the efficiency of UADLLME. Extraction conditions: concentration of analytes, 100 μg/L; sample volume, 5.0 mL; extraction solvent, 1,1,2,2-tetrachloroethane; volume of extraction solvent, 60 μL; dispersive solvent, methanol; dispersive solvent volume, 200 μL; centrifugation time, 5 min; addition of NaCl, 2% (m/v); room temperature.

3.1.7. Effect of centrifugation time

During UADLLME methodology, ultrasonic agitation causes the extractant to completely disperse throughout the aqueous phase and to form vast organic vesicles to achieve efficient extraction [26]. Centrifugation was used to break down the cloud solution and to deposit the sediment phase in the extraction tubes. The effect of centrifugation time on the extraction efficiency in the range of 5–25 min was assayed at 3500 rpm. The extraction efficiency for the analytes was lower when the centrifugation time was too short while longer centrifugation times had no significant effect on the extraction efficiency. Therefore, 10 min was adopted as the centrifugation time for treatment of the samples in this study to obtain a separated biphasic system with the highest possible recovery and efficiency.

3.2. Data analysis

To investigate the effect of factor optimization for each metabolite and to evaluate which factor had the central influence, a new data analysis method named fitting derivation method was introduced for the first time. This new strategy is much simple and convenient as it can be operated without costly statistical softwares such as STATISTICA and MINILAB. The effects of volume of 1,1,2,2-tetrachloroethane, volume of methanol, concentration of NaCl (w/v), ultrasonic treatment time and centrifugation time were studied as the influences of the factors could be quantified. Each level of these factors was designated as 1–5, except for the volumes of 1,1,2,2-tetrachloroethane, which were termed 1–6 (where 1–6 stands for 40–90 μL, respectively). Curve fitting these factors against recoveries was easily performed using MS office software with a polynomial curve fitting equation (ensuring that all the R^2 values in these curves were higher than 0.999). The second step was to take the derivatives of the curves' functions and obtain the corresponding derived functions. Finally, the derivatives were calculated at the optimized point according to each factor's derived functions, and the resulting absolute value was taken. It is well known that the derivative is a measure of how a function changes as its input changes, and in this study, a larger absolute derivative value indicates that changing that factor has a larger influence on the observed experimental recovery. According to Table 1, changing the volumes of 1,1,2,2-tetrachloroethane and methanol as well as varying the concentration of NaCl had the greatest impact on the extraction of 1,4-BDM. On the other hand, 1-DM recovery was more strongly influenced by prolonged periods of ultrasonic

Table 1
Fitting derivation results.

		MEQE	4-DM	1-DM	1,4-BDM	Average absolute derivatives
Volume of extraction solvent	Fitting formula	$f(x) = -0.647x^5 + 11.92x^4 - 82.23x^3 + 259.5x^2 - 360.0x + 209$	$f(x) = -0.559x^5 + 10.50x^4 - 74.09x^3 + 239.6x^2 - 342.9x + 225.2$	$f(x) = -0.495x^5 + 9.308x^4 - 65.84x^3 + 214.0x^2 - 309.0x + 214.4$	$f(x) = -0.479x^5 + 9.225x^4 - 66.87x^3 + 222.5x^2 - 327.1x + 235.2$	–
	Derivative formula	$f'(x) = -3.235x^4 + 47.68x^3 - 246.69x^2 + 519x - 360$	$f'(x) = -2.795x^4 + 42x^3 - 222.27x^2 + 479.2x - 342$	$f'(x) = -2.475x^4 + 37.232x^3 - 197.52x^2 + 428x - 309$	$f'(x) = -2.395x^4 + 36.9x^3 - 200.61x^2 + 445x - 327.1$	–
	Absolute derivative at optimized point	2.115	2.775	2.109	4.715	2.929
Volume of dispersive solvent	Fitting formula	$f(x) = 0.537x^4 - 6.825x^3 + 30.81x^2 - 61.12x + 90.7$	$f(x) = 0.195x^4 - 2.725x^3 + 14.20x^2 - 34.67x + 92.5$	$f(x) = 0.404x^4 - 5.491x^3 + 26.84x^2 - 56.35x + 107.3$	$f(x) = 0.745x^4 - 9.641x^3 + 44.25x^2 - 86.45x + 137$	–
	Derivative formula	$f'(x) = 2.184x^3 - 20.475x^2 + 61.62x - 61.12$	$f'(x) = 0.776x^3 - 8.175x^2 + 28.4x - 34.67$	$f'(x) = 1.616x^3 - 16.473x^2 + 53.68x - 56.35$	$f'(x) = 2.98x^3 - 28.923x^2 + 88.5x - 86.45$	–
	Absolute derivative at optimized point	17.791	13.669	17.527	23.893	18.220
Concentration of NaCl (w/v)	Fitting formula	$f(x) = -1.05x^4 + 13.15x^3 - 57.35x^2 + 100.2x - 3.1$	$f(x) = -1.433x^4 + 17.95x^3 - 77.91x^2 + 132.1x - 4.1$	$f(x) = -1.383x^4 + 17.43x^3 - 76.06x^2 + 128.5x + 1.2$	$f(x) = -1.770x^4 + 22.80x^3 - 100.6x^2 + 168.7x - 6.9$	–
	Derivative formula	$f'(x) = -4.2x^3 + 39.45x^2 - 114.7x - 100.2$	$f'(x) = -5.732x^3 + 53.85x^2 - 155.82x - 132.1$	$f'(x) = -5.532x^3 + 52.29x^2 - 152.12x - 128.5$	$f'(x) = -7.08x^3 + 68.4x^2 - 201.2x - 168.7$	–
	Absolute derivative at optimized point	5.000	10.196	10.836	16.740	10.693
Ultrasonic time	Fitting formula	$f(x) = -0.487x^4 + 5.991x^3 - 25.66x^2 + 45.55x + 29.8$	$f(x) = -0.637x^4 + 7.675x^3 - 32.11x^2 + 54.77x + 41.3$	$f(x) = -0.95x^4 + 11.1x^3 - 44.25x^2 + 70.6x + 37.7$	$f(x) = -0.725x^4 + 8.366x^3 - 33.22x^2 + 53.08x + 54.1$	–
	Derivative formula	$f'(x) = -1.948x^3 + 17.973x^2 - 51.32x + 45.55$	$f'(x) = -2.548x^3 + 23.025x^2 - 64.22x + 54.77$	$f'(x) = -3.8x^3 + 33.3x^2 - 88.5x + 70.6$	$f'(x) = -2.9x^3 + 25.098x^2 - 66.44x + 53.08$	–
	Absolute derivative at optimized point	3.166	3.218	6.200	3.288	3.968
Centrifugation time	Fitting formula	$f(x) = -0.720x^4 + 9.791x^3 - 47.47x^2 + 96.70x + 1.3$	$f(x) = -0.641x^4 + 8.766x^3 - 42.45x^2 + 85.43x + 24$	$f(x) = -0.645x^4 + 8.825x^3 - 43.70x^2 + 91.82x + 20.7$	$f(x) = -0.408x^4 + 5.316x^3 - 24.49x^2 + 46.98x + 57$	–
	Derivative formula	$f'(x) = -2.88x^3 + 29.373x^2 - 94.94x + 96.70$	$f'(x) = -2.564x^3 + 26.298x^2 - 84.9x + 85.43$	$f'(x) = -2.58x^3 + 26.475x^2 - 87.4x + 91.82$	$f'(x) = -1.632x^3 + 15.948x^2 - 48.98x + 46.98$	–
	Absolute derivative at optimized point	1.272	0.310	2.280	0.244	1.027

Table 2
The performance characteristics of the UADLLME method combined with HPLC-VWD.

Metabolites	Linearity equation	Linearity (μg/L)	*r*	RSD (%)	Enrichment factors	LOD (μg/L)	LOQ (μg/L)	Recovery (%)
MEQE	$Y = 1132.9X + 8.5911$	0.5–500	0.9957	4.6	75	0.16	0.52	72.0%
4-DM	$Y = 1235.5X + 7.8504$	0.5–500	0.9968	4.1	88	0.24	0.81	85.3
1-DM	$Y = 1703.5X + 10.601$	0.5–500	0.9976	4.3	93	0.24	0.79	89.8
1,4-BDM	$Y = 1316.8X + 9.4110$	0.5–500	0.9970	5.2	95	0.28	0.94	91.3

RSD: relative standard deviation; LOD: limits of detection (S/N = 3); LOQ: limits of quantitation (S/N = 10).

Table 3
Comparison of UADLLME with other methods for the quantitation of QdNOs derivatives.

Method	Extraction time (min)	Analytical ranges	LODs	Recovery	Ref.
SPE-HPLC	30	2–100 μg/kg	0.7–5.6 μg/kg	59–77%	[28]
USE-SPE-HPLC	20	0.2–50 mg/kg	0.07–0.66 mg/kg	75–104%	[12]
MSPD-UHPLC	30	0.02–10 mg/L	0.02–0.10 mg/kg	89–98%	[29]
UADLLME-HPLC	4	0.5–500 μg/L	0.16–0.28 μg/L	72–91%	Present work

SPE, solid phase extraction; USE, ultrasonic solvent extraction; MSPD, matrix solid-phase dispersion; UHPLC, ultra-high-pressure liquid chromatography.

Table 4
Analytical results in pig urine samples.

Metabolites	Spiked (μg/L)	Urine sample 1			Urine sample 2		
		Measured (μg/L)	Recovery (%)	RSD (%)	Measured (μg/L)	Recovery (%)	RSD (%)
MEQE	50.0	35.6	71.2	4.5	35.4	70.8	4.7
	100.0	72.5	72.5	5.1	73.1	73.1	5.2
4-DM	50.0	41.0	82.0	4.6	41.7	83.4	5.4
	100.0	82.2	82.2	5.2	82.8	82.8	5.7
1-DM	50.0	44.3	88.5	3.6	44.8	89.6	4.3
	100.0	88.3	88.3	4.8	87.9	87.9	5.5
1,4-BDM	50.0	46.6	93.1	2.9	46.0	92.0	3.4
	100.0	93.2	93.2	4.5	91.5	91.5	5.0

treatment and centrifugation. Average absolute derivatives (ADs) of all four metabolites for each factor were used to evaluate the central influence. Calculated AD values indicated that the volume of dispersive solvent was the central influence in this study, followed by the concentration of NaCl and ultrasonic treatment time. Centrifuging time seems to have less effect on the extraction efficiency. This is probably because the effect of no centrifugation is not being taken into account and because once the biphasic system is separated, extra centrifuging time is of little significance.

3.3. Analytical performance of the proposed method

The optimum conditions selected for UADLLME were as follows: 5.0 mL sample solution with 2% salt addition, 60 μL 1,1,2,2-tetrachloroethane as extraction solvent, 200 μL methanol as the dispersive solvent, 4 min ultrasonic treatment time, and 10 min centrifugation at 3500 rpm. Under the above-mentioned optimal conditions, a series of experiments were designed to obtain linear calibration ranges, regression equations, limits of detection (LODs), limits of quantitation (LOQs) and other characteristics of the method. Three replica extractions were performed for each concentration level. The results are shown in Table 2. The calibration curve was linear in the range of 0.5–500 μg/L for MEQE, 4-DM, 1-DM and 1,4-BDM with the correlation coefficients ranging from 0.9957 to 0.9976. The LOD was calculated from triple signal-to-noise (S/N = 3) and gave values between 0.16 and 0.28 μg/L. Meanwhile, the LOQ was calculated from 10 times signal-to-noise (S/N = 10) to be 0.52–0.94 μg/L. The RSDs of the insecticides ranged from 4.1% to 5.2%, and the extraction recoveries and enrichment factors of this method were acceptable, ranging from 72.0% to 91.3% and 75–95, respectively. By calculated using chemaxon.marvin.calculations.logPPlugin [27], the octanol–water

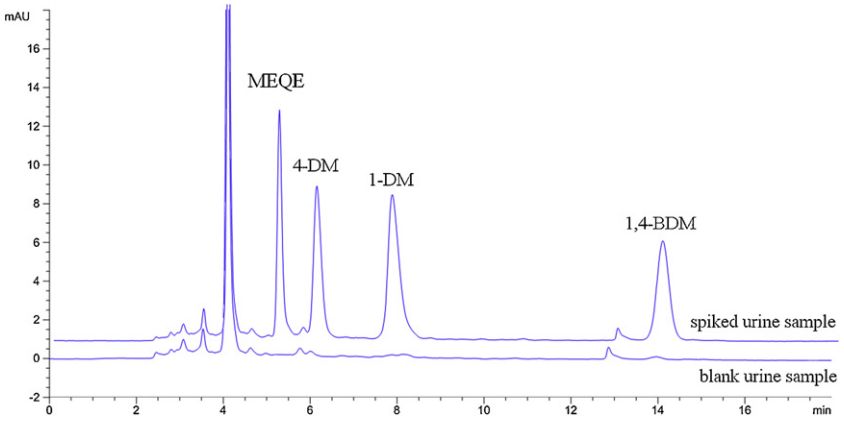

Fig. 8. Typical chromatograms of 1-DM, 4-DM, 1,4-BDM and MEQE in spiked (100.0 μg/L) and blank pig urine sample.

partitioning coefficient (K_{ow}) of MEQE was 9.77 and $\log K_{ow}$ was 0.99. Probably due to its smaller $\log K_{ow}$, it was difficult to be extracted by 1,1,2,2-tetrachloroethane from the water samples and resulted not very satisfied recovery of MEQE.

As there is no published report for simultaneously measuring the levels of mequindox metabolites, a comparison was made between the presented method and different methods for the determination of related QdNOs derivatives. The results are summarized in Table 3. The UADLLME methodology proposed in this work is shown to be superior in a number of ways: (i) the simple operation procedure makes the sample preparation easy, and the ultrasound-assisted procedure makes the extraction rapid; (ii) dispersive liquid–liquid microextraction reduces the consumption of organic solvents, and dispersion by ultrasound-assisted processing further enhances this effect; and (iii) a small sample volume is adequate for analysis owing to the high enrichment factors and appropriate recoveries. In conclusion, the presented method is highly efficient and has the potential to become the most-used analytical method for the preconcentration and detection of QdNOs derivatives in liquid samples.

3.4. Real samples analysis

To evaluate the applicability and accuracy of the proposed method in real samples, two pig urine samples from female changbai pigs were collected and analyzed. The samples were pretreated as described in Section 2.3, extracted using the described UADLLME methodology and analyzed by HPLC-VWD. In addition, a urine sample was selected as a matrix, and analytes were added to it at two different levels; this 'spiked' sample was then also analyzed using the described UADLLME methodology. The analytical results are shown in Table 4, and typical chromatograms from both the blank and the spiked samples are shown in Fig. 8. The average recoveries for 1-DM, 4-DM, 1,4-BDM and MEQE were in a range of 71.9–93.2% with a RSD of less than 5.7% ($n = 3$), which indicated that the method was reliable and could be used for the identification of traces of QdNOs derivatives in biological fluid samples.

4. Conclusion

In the present study, a simple, rapid and inexpensive UADLLME recovery and concentration method was developed and combined with HPLC-VWD for the simultaneous identification of four synthesized metabolites of mequindox. The optimum parameters for extraction performance have been evaluated. By using a fitting derivation method, the effect of factor optimization on detection of each metabolite was investigated. Good linearity was observed in a range of 0.5–500 μg/L with a LOD of 0.16–0.28 μg/L (S/N = 3) and a LOQ of 0.52–0.94 μg/L (S/N = 10). The recoveries of the compounds studied ranged from 72.0% to 91.3% with a RSD of less than 5.2% and an EF in a range of 75–95. The performance of this procedure in the extraction of 1-DM, 4-DM, 1,4-BDM and MEQE from pig urine samples was satisfactory. In addition, adequate repeatability, reproducibility, linearity and the absence of matrix effects demonstrated that UADLLME seems to have potential for the analysis of other QdNOs derivatives in urine samples.

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