Determination of Thiamphenicol in Honey by Dispersive Liquid–Liquid Microextraction with High-Performance Liquid Chromatography

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Abstract

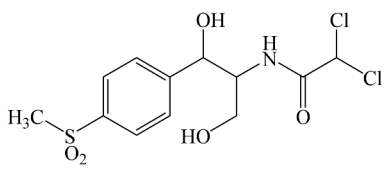
Dispersive liquid–liquid microextraction (DLLME) coupled with high-performance liquid chromatography–variable wavelength detector (HPLC–VWD) was developed for extraction and determination of thiamphenicol (THA) in honey. A mixture of extraction solvent (30 µL 1,1,2,2-tetrachloroethane) and dispersive solvent (1.0 mL of acetonitrile) was rapidly injected into 5.00 mL sample solution for the formation of cloudy solution. The analyte in the sample was extracted into the fine droplets of $C_2H_2Cl_4$. After extraction, phase separation was performed by centrifugation, and the enriched analyte in the sedimented phase was determined by HPLC–VWD. Some important parameters, such as the kind and volume of extraction solvent and dispersive solvent, extraction time, sample solution pH, sample volume, and salt effect, were investigated and optimized. Under the optimum extraction condition, the method yielded a linear calibration curve in the concentration range from 3 to 2000 µg/kg for target analyte. The enrichment factors for THA was 87.9, and the limit of detection (S/N = 3) was 0.1 µg/kg. The relative standard deviation for the extraction of 10 µg/kg of THA was 6.2% (n = 6). The main advantages of DLLME-HPLC method are simplicity of operation, rapidity, low cost, high enrichment factor, high recovery, good repeatability, and extraction solvent volume at the µL level. Honey samples were successfully analyzed using the proposed method.

Introduction

Thiamphenicol (THA) is an analogue of chloramphenicol in which the nitro group in the benzene ring is replaced with a methylsulfonic group (Figure 1). It was reported that THA shows particular therapeutic effect in respiratory infections, bacterial prostatitis, and venereal diseases. But THA also shows haematological toxicity (1). So, it is very important to develop a sensitive, rapid, and simple method for the determination of THA in food commodities.

Up to now, the main approaches for the detection of THA residue include chromatography (2) and multiple techniques of chromatography linked with mass spectrum (3–6) using liquid–liquid extraction (LLE) or solid-phase extraction (SPE) techniques for preconcentration of THA, respectively. However, these methods have their own disadvantages, such as complex analyzing processes, long time requirement for preparation of samples, and expensive equipments. The difficulty in determining THA in foods is the extremely low concentrations of 1–10 µg/kg in various samples with complex matrices. Therefore, novel, rapid, and accurate clean-up and enrichment methods are required for analyses involving THA monitoring.

SPE is routinely used for clean-up and preconcentration in the analysis of biological and environmental samples (7). Compared with LLE, SPE has the advantages of simplicity, speed, and less consumption of organic solvents. However, generic sorbents usually lack selectivity and are easily subjected to interference by non-target substances with similar characteristics (8). Although immunoaffinity chromatography (IAC) is capable of differentially adsorbing target analytes, it still has some disadvantages such as lack of stability and high costs of antibody preparation. Recent research has been oriented towards the development of efficient, economical, and miniaturized sample preparation methods. As a result, solid-phase microextraction (SPME) (9,10) and liquid-phase microextraction (LPME) (11) have been developed. Compared with LLE, SPME is a solvent-free process that includes simultaneous extraction and preconcentration of analytes from aqueous samples or the headspace of the samples. However, SPME is expensive, its fiber is fragile and has limited lifetime, and sample carry-over could be a problem (12). LPME was developed as a



Thiamphenicol (THA)

Figure 1. Structural formula of THA.

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solvent-minimized sample pretreatment procedure that is inexpensive, and because very little solvent is used, there is minimal exposure to toxic organic solvents (13,14). However, this method suffers from some disadvantages as follows: fast stirring would tend to format air bubble (5), extraction is time-consuming, and equilibrium could not be attained after a long time in most cases (15).

Recently, a novel microextraction technique termed as dispersive liquid–liquid microextraction (DLLME) has been developed for the determination of polycyclic aromatic hydrocarbons in water by Assadi and co-workers (16). It is based on a ternary component solvent system like homogeneous liquid–liquid extraction and cloud point extraction. In this method, the appropriate mixture of extraction solvent and dispersive solvent is injected into aqueous sample rapidly by syringe, and a cloudy solution is formed. The analyte in the sample is extracted into the fine droplets of extraction solvent. After extraction, phase separation is performed by centrifugation, and the enriched analyte in the sedimented phase is determined by chromatography or spectrometry methods. The advantages of the DLLME method are simplicity of operation, rapidity, low cost, high recovery, and enrichment factors. This method has been applied for the determination of trace organic pollutants and metal ions in the environmental samples (17–22).

In this study, DLLME followed by high-performance liquid chromatography (HPLC) with variable wavelength detector (VWD) has been investigated for the determination of THA in honey samples. The effects of various experimental parameters, such as the kind and volume of extraction solvent and dispersive solvent, extraction time, sample solution pH, and salt effect, were studied and optimized. The optimized method was applied to determine THA in honey samples to evaluate the application of this method to real samples.

Experimental

Reagents and standards

Thiamphenicol (99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol and acetonitrile were were purchased from Fisher (Walham, MA). Chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), dichloromethane ($C_2H_4Cl_2$), 1,1,2,2-tetrachloroethane ($C_2H_2Cl_4$), acetone, and sodium chloride were all analytical-grade. The water used was purified on an Ultrapure Water System (Millipore, Beijing, China).

The stock standard solution was prepared in methanol at a concentration of 300 µg/mL and stored at 4°C in refrigerator. Working standard solutions of THA was prepared by appropriate dilution of the stock solution using deionized water.

One gram of honey was weighed into a 10-mL centrifuge tube with a conical bottom, 5.0 mL of water was added, and the mixture was vortexed until a homogeneous sample was obtained. Then, the homogeneous sample was used for DLLME-HPLC analysis directly.

Instrumentation

The chromatographic analysis was performed on a Dionex Summit P680 HPLC system equipped with a manual injector and a VWD (Sunnyvale, CA). A personal computer equipped with a Dionex Chromeleon ChemStation program for LC was used to process chromatographic data. A Varian Pursuit-C_{18} column (5 µm, 4.6 mm × 250 mm) was connected with a guard column (cartridge 2.1 × 12.5mm, 5 µm) (Agilent, Santa Clara, CA) filled with the same packing material for separations. The mobile phase was a mixture of methanol–water (55:45, v/v), and the flow rate was 1.0 mL/min. The column temperature was set at 25ºC, and the VWD detector was set at a wavelength of 225 nm. All injections were performed manually with a 5.0-µL sample loop. An 80-2 centrifuge (Jiangshu Zhongda Electric Appliance, Jiangsu, China) was used for centrifuging.

Extraction procedure

For the DLLME, an aliquot of 5.00 mL of working standard solution (300 ng/mL of THA) was placed in a 10-mL glass test tube with conical bottom. One milliliter of acetonitrile (as dispersive solvent) containing 30 µL $C_2H_2Cl_4$ (as extraction solvent) was injected rapidly into the sample solution by using 1.00 mL syringe, and then the mixture was gently shaken by hands for several seconds. A cloudy solution that consists of very fine droplets of $C_2H_2Cl_4$ dispersed into the sample solution was formed, and the analyte was extracted into the fine droplets. After centrifugation for 2 min at 4000 rpm, the extraction solvent was sedimented in the bottom of the conical test tube (about 35 µL). An aliquot of 30 µL of sedimented phase was removed using a 100-µL HPLC microsyringe and injected into the HPLC system for analysis. All experiments were performed in duplicate, and means of results were used in plotting of curves.

Results and Discussion

Optimization of DLLME

In this study, DLLME combined with HPLC was used for preconcentration and determination of THA in honey samples. Several parameters that influence the extraction efficiency had to be studied and optimized. Such parameters included the nature and volume of extraction solvent, the nature and volume of dispersive solvent, the extraction time, the volume and pH of sample solution, and the effect of salt concentration.

In order to obtain the optimized extraction conditions, enrichment factor (EF) and extraction recovery (ER) were used to evaluate the extraction efficiency under different conditions. The enrichment factor was defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration of analyte (C_0) within the sample (16):

$$EF = \frac{C_{sed}}{C_0}$$

The C_{sed} was obtained from calibration graph of direct injection of chloramphenicol standard solution in the extraction solvent. The extraction recovery was defined as the percentage

of the total analyte amount (n_0), which was extracted to the sedimented phase (n_{sed}).

$$ER = \frac{n_{sed}}{n_0} \times 100 = \frac{C_{sed} \times V_{sed}}{C_0 \times V_{aq}} \times 100$$

$$ER = \frac{V_{sed}}{V_{aq}} EF \times 100$$

where V_{sed} and V_{aq} are the volumes of sedimented phase and sample solution, respectively.

Selection of extraction solvent

The selection of an appropriate extraction solvent is of high important for the DLLME process. In the selection of extraction solvent, some properties must be considered such as (A) good chromatographic behavior under the selected HPLC conditions, (B) higher density than water, (C) extraction capability of target compounds, (D) low solubility in water, and (E) can form a stable two-phase system in the presence of a dispersive solvent when injected to an aqueous solution. Among the solvents with density higher than water (mainly chlorinated solvents), CH_2Cl_2 (1.32 g/mL), $CHCl_3$ (1.47 g/mL), CCl_4 (1.59 g/mL), and $C_2H_2Cl_4$ (1.54 g/mL) were studied. On the other hand, the selection of a dispersive solvent is limited to solvents such as methanol, acetonitrile, and acetone, which are miscible with both water and extraction solvents. In this study, all combinations of CH_2Cl_2, $CHCl_3$, CCl_4, and $C_2H_2Cl_4$ (30.0 μL) as extraction solvents and methanol, acetonitrile, and acetone (1.00 mL) as dispersive solvents were tested. In the case of CH_2Cl_2 and $C_2H_4Cl_2$ as extraction solvents, a two-phase system was not observed with any studied dispersive solvents when they were injected to 5.00 mL analytes solution in water. In the case of $CHCl_3$ as extraction solvent, the two-phase system was not stable. In the case of CCl_4 as extraction solvent, no chromatographic peak can be found. For $C_2H_2Cl_4$, a two-phase system was formed stably with all three dispersive solvents, and its sedimented phase can easily be removed by microsyringe to be introduced into the HPLC and has less consumption volume. So $C_2H_2Cl_4$ was selected as the extraction solvent.

Selection of dispersive solvent

The miscibility of the disperser solvent in the extraction solvent and aqueous phase is the most important factor affecting the selection of disperser solvent in DLLME. These solvents can disperse extraction solvent as very fine droplets in aqueous phase. Methanol, acetonitrile, and acetone have this property and were selected for this purpose. The experiments were performed by using 1.00 mL of each dispersive solvent containing 30.0 µL $C_2H_2Cl_4$, and three replicate tests were performed for each type of dispersive solvent. The results indicated that acetonitrile exhibited the highest extraction efficiency. For acetone, the solvent peak was high enough to interfere with the analyte peak. Thus, acetonitrile was chosen as the dispersive solvent for subsequent experiments.

Effect of extraction solvent volume

In order to study the effect of extraction solvent volume on the extraction efficiency, different volumes of $C_2H_2Cl_4$ (10.0–80.0 µL at 10.0 µL interval) and a constant volume of acetonitrile (1.00 mL) were tested. Figures 3–5 show curves of volume of sedimented phase, EF, and ER versus volume of extraction solvent, respectively. As can be seen, by increasing the volume of extraction solvent from 10.0 to 80.0 µL, the volume of sedimented phase increases from 16.0 to 108.0 µL, but EF decreases because the volume of sedimented phase

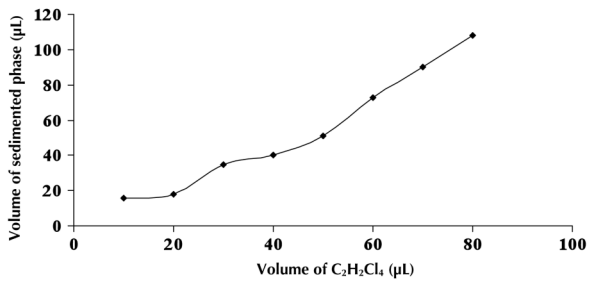

Figure 2. Effect of the volume of extraction solvent ($C_2H_2Cl_4$) on the volume of sedimented phase obtained by DLLME. Extraction conditions: sample volume, 5.00 mL; dispersive solvent (acetonitrile) volume, 1.00 mL; room termperature.

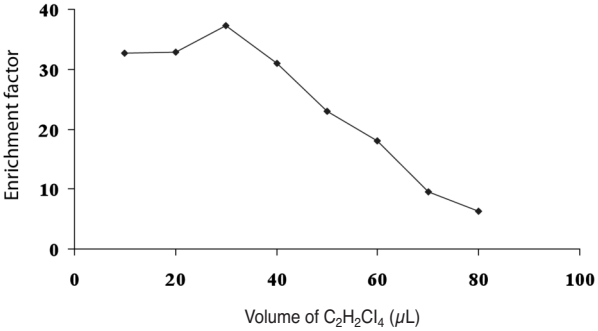

Figure 3. Effect of the volume of extraction solvent ($C_2H_2Cl_4$) on the enrichment factor of THA extracted by DLLME. Extraction conditions: sample volume, 5.00 mL; dispersive solvent (acetonitrile) volume, 1.00 mL; room termperature.

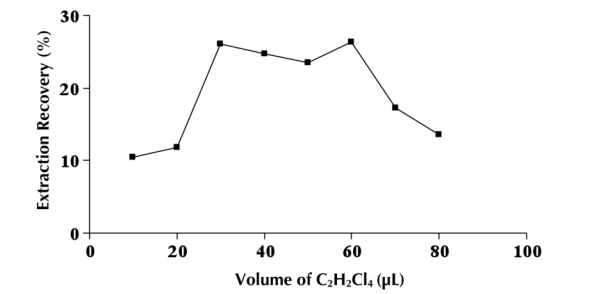

Figure 4. Effect of the volume of extraction solvent ($C_2H_2Cl_4$) on the recovery of THA obtained by DLLME. Extraction conditions: same as in Figure 2.

increases. ER increases at first, then decreases. On the basis of these results, 30.0 µL of $C_2H_2Cl_4$ was selected for subsequent experiment.

Effect of dispersive solvent volume

The enrichment factor is very difficult to investigate because variation of the volume of acetonitrile (dispersive solvent) changes the volume of sedimented phase for a constant volume of $C_2H_2Cl_4$ (extraction solvent) (15). To avoid this problem, the volume of acetonitrile and $C_2H_2Cl_4$ should be changed simultaneously to achieve a constant volume of sedimented phase (35.0 µL). Various experiments were performed by using different volumes of acetonitrile (0.50, 0.75, 1.00, 1.25, and 1.50 mL) containing 27.0, 28.0, 30.0, 31.6, and 33.0 µL $C_2H_2Cl_4$, respectively. The results were shown in Figure 5. As can be seen, EF increased with the increase of the volume of acetonitrile when it less than 1.00 mL. Reduction in EF was observed after the volume of acetonitrile exceeded 1.00 mL. This is because at low volume, acetonitrile cannot disperse $C_2H_2Cl_4$ properly, and cloudy solution is not formed completely; and at high volume, the solubilities of THA in water increase. So 1.00 mL acetonitrile was chosen as optimum volume.

Effect of extraction time

In DLLME, extraction time is defined as interval time between injecting the mixture of disperser solvent (acetonitrile) and extraction solvent ($C_2H_2Cl_4$), and before starting to centrifuge. The effect of extraction time was examined in the range of 0–60 min with constant experimental conditions. The obtained results showed that the variations of EF and ER versus extraction time are not remarkable. It is revealed that the DLLME method is time-independent because of the infinitely large surface area between extraction solvent and aqueous phase after the formation of cloudy solution lead to the transition of analytes from aqueous phase to extraction solvent is very fast, and equilibrium state is achieved quickly. This is the most important advantage of DLLME technique. In this method, the most time-consuming step is the centrifuging of sample solution in the extraction procedure, which is about 2 min.

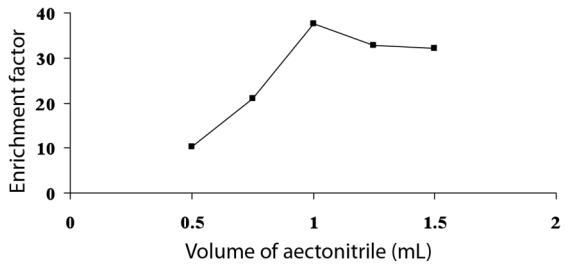

Figure 5. Effect of the volume of dispersive solvent (acetonitrile) on the enrichment factor of THA obtained by DLLME. Extraction conditions: sample volume, 5.00 mL; sedimented phase volume, 35.0 µL; room temperature.

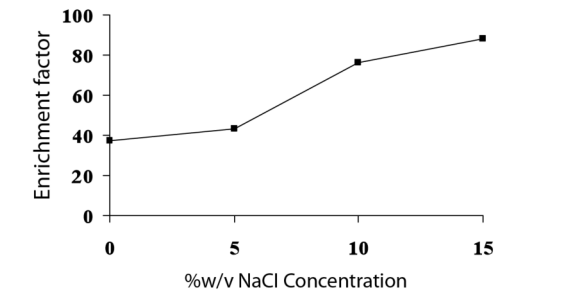

Figure 6. Effect of salt concentration on enrichment factor of THA obtained by DLLME. Extraction conditions: sample volume, 5.00 mL; dispersive solvent (acetonitrile) volume, 1.00 mL; extraction solvent ($C_2H_2Cl_4$) volume, 30.0 µL.

Effect of ionic strength

For investigating the influence of ionic strength on the extraction efficiency of DLLME, various experiments were performed by adding different amounts of NaCl (0–30% w/v) with other experimental conditions, which were kept constant. It was found that the extraction solvent ($C_2H_2Cl_4$) floated upon aqueous solution of sample after extraction and centrifugation when adding 20–30% (w/v) of NaCl to sample solution. The volume of the sedimented phase increases from 35.0 to 68.0 μL by increasing the amount of NaCl from 0 to 15% because the solubility of extraction solvent in aqueous phase decreases.

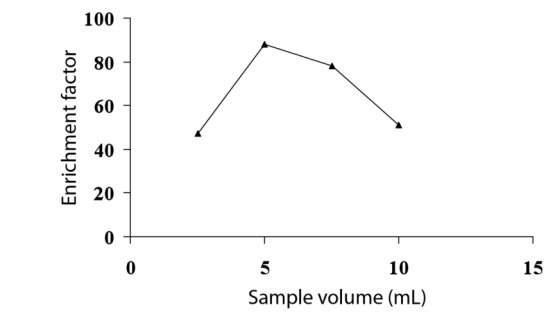

Figure 7. The effect of the sample volume on the enrichment factor of THA obtained by DLLME. Extraction conditions: room temperature.

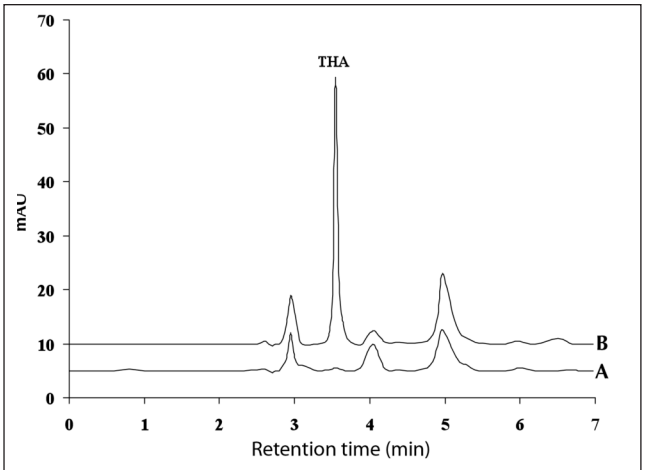

Figure 8. HPLC chromatogram of homogeneous honey sample spiked with selected THA at concentraction level 300 µg/kg (A) before performing DLLME and (B) after performing DLLME. Extraction conditions: sample volume, 5.00 mL; extraction solvent, 30.0 µL tetrachloroethane; dispersive solvent 1.00 mL acetonitrile.

The results (Figure 6) reveal that the enrichment factor increased significantly with an increase of salt concentration. Based on these results, 15% w/v NaCl was chosen as the optimal salt concentration in the DLLME procedure.

Effect of the sample solution pH

The solution pH of the sample is a significant factor, which may affect the extraction recovery of THA in water samples. When the pH changes, the acid-base equilibrium for THA shifts significantly toward the neutral forms or ionic forms. The effect of the sample solution pH on the THA extraction from water samples was studied within the range of 2–7 using H_3PO_4. The results indicate that the enrichment factor remained constant with the pH increase from 2 to 7. THA is not stable when the sample solution pH > 7. Thus, the neutral solution was chosen as optimum pH because it is simple to prepare the sample solution.

Study of sample volume

For this purpose 2.50, 5.00, 7.50, and 10.0 mL analyte solutions (containing 300 µg/mL of THA) were selected as sample size and the DLLME procedure using acetonitrile as dispersive solvent (0.50, 1.00, 1.50, and 2.00 mL, respectively) and 1,1,2,2-tetrachloroethane as extraction solvent was performed. The results show that by increasing sample volume, the sedimented phase volume (65.0, 35.0, 22.0, and 15.0 µL) decreased. The EF for analytes also changed (Figure 7). Thus 5.00 mL analyte solution was used in DLLME procedure.

Evaluation of DLLME method

After optimizing all extraction conditions, the best conditions have been selected to evaluate the performance of the method: 30.0 µL of $C_2H_2Cl_4$ as extraction solvent, 1.00 mL acetonitrile as dispersive solvent, 15% w/v NaCl, neutral sample solution, and 5.00 mL sample solution.

Chromatograms of honey sample after spiking of THA at the concentration level 300 µg/kg of analyte along with concentrated solution under the optimum conditions are shown in Figure 8. The chromatograms were characterized by symmetrical peak shape.

Features of the method

Under the optimum conditions, using blank honey samples spiked at different concentrations of analyte, linearity was observed over the range 3–2000 µg/kg with a correlation coefficient (R^2) of 0.9992 for THA, respectively. Limits of detection (LOD), on the basis of three times the signal-to-noise ratio (S/N), was 0.1 µg/kg. The limit of quantification (LOQ), defined as the lowest studied concentration with acceptable precision and accuracy, was 1.2 µg/kg. The precision of this method was determined by successive six-time analysis of blank honey sample spiked at 10 µg/kg analyte; the relative standard deviation (RSD) was 6.2%; the inter-day precision (RSD) was 9.1%. The enrichment factor was 87.9.

Real samples analysis

In order to investigate the applicability of the proposed methods, three honey samples from supermarket were analyzed using the proposed method. The results showed that the analyzed samples were free of THA. To test the applicability and accuracy of the proposed method in real samples analysis, a honey sample was selected as matrix, analyte was added to it in three levels, and the DLLME method was performed. The results showed that recoveries, defined as the percentage ratio between concentration of THA found and concentration of THA added, from the honey sample were from 89.7% to 93.6% with RSD (n = 6) less than 6.3%. This indicated that matrix does not influence the proposed DLLME method for preconcentration of THA from honey samples. So, the DLLME-HPLC–VWD method is feasible for quantitative analysis of CAP and THA in real samples and could be used in routine analysis.

Conclusion

A simple, rapid, and inexpensive DLLME recovery and concentration technique has been coupled to HPLC–VWD method for the determination of THA in honey samples. The optimum conditions of extraction performance have been obtained. The experimental results reveal that this method provides high enrichment factor within a short time, lower solvent consumption, higher enrichment factor, good linearity over the investigated concentration range, and high quantitative recovery. The performance of this procedure in the extraction of THA from honey samples was satisfactory. Comparison of this new method with other extraction methods such as LLE, SPE, SPME, and LPME shows that DLLME is advantageous in terms of total extraction time, cost, and feasibility.

Acknowledgments

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