RESEARCH ARTICLE

Functionalized Multi Walled Carbon Nanotubes-Reinforced Hollow Fiber Solid/Liquid Phase Microextraction and HPLC-DAD for Determination of Phenazopyridine in Urine


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Abstract: Introduction: A sensitive analytical method based on functionalized multi walled carbon nanotubes reinforced hollow fiber solid/liquid phase microextraction (F-MWCNTs-HF-SLPME) forwarded with HPLC-DAD for analyzing phenazopyridine from urine is presented.

Materials and Methods: The extraction of phenazopyridine is performed using specially designed F-MWCNTs-HF-SLPME device constructed as follows: the functionalized multi walled carbon nanotubes (F-MWCNTs) were immobilized into the pores of 2.5 cm hollow fiber micro-tube using capillary forces and ultrasonication, then, the lumen of the micro-tube was filled with 1-octanol with two ends sealed. Subsequently, the device was placed into 10-mL of urine sample containing the analyte with agitation. After ending extraction, the device was removed, rinsed, sonicated in 250 µL of organic solvent and analyzed directly by the separation system.

Conclusion: Different parameters affecting the performance of the developed method were optimized. The method showed good linearity with (R²) 0.999 and good repeatability with (RSDs) from 3.7 to 0.9% at analyte concentration ranged from 0.01 to 10 µg L⁻¹ of spiked urine samples. The limit of detection/ quantitation, LODs/LOQs was 0.02/0.09 µg L⁻¹. In comparison with reference methods, the developed method is considered as a promising microextraction technique for determination of trace phenazopyridine in human urine using a common HPLC without further cleanup procedures.

Keywords: Functionalized multi-walled carbon nanotubes, liquid chromatography, phenazopyridine, reinforced solid/liquid phase microextraction, Urine.

1. INTRODUCTION

Phenazopyridine (PHP), which is 3-Phenylazo-pyridine-2,6-diamine (Fig. 1), is mainly prescribed as analgesic for urinary tract infection in conjunction with other medication to give immediate and symptomatic relief for patients[1-3]. PHP is available as an over-the-counter drug [4,5]. However, a common and harmless side effect of this drug is the special color change (dark orange to reddish) in the urine and sometimes a remarkable yellowish color change in the skin or eyes [6]. Moreover, fever, confusion, skin rash, shortness of breath and swelling of the face, fingers, feet, or legs can also be observed [7] and therefore, it is important for physicians to be aware of the toxicity of this commonly used drug and should look closely for symptoms of renal insufficiency [8].

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Fig. (1). Structure of PHP.
A literature review shows that several methods have been developed for the quantification of PHP including: chromatographic method with UV detection (HPLC-UV) [9-11], electroanalytical methods (such as voltammetry [7], and potentiometry [12]) and flow injection analysis [13]. Additionally, liquid and gas chromatography with mass spectrometry have also been used for analysis and pharmacokinetic study of this drug [1,14]. Nevertheless, the direct determination of trace PHP levels in urine is difficult due to the low concentration of PHP (sometimes, even lower than the detection limit [2]) with serious interference of sample matrix. Thus, extra sample separation and pre-concentration approaches are needed to simultaneously remove the sample matrix and increase the concentration level of analyte.

Liquid phase microextraction (LPME) has attracted the increasing attention in the development of simple and environmentally friendly analytical microextraction procedures [15-17]. As one of the operating modes of LPME, liquid phase microextraction using hollow fiber membranes (HF-LPME) has been reported [18-20].

HF-LPME technique in which analytes are extracted into a supported liquid membrane (SLM) immobilized into the pores of hollow fiber (HF), then forwarded into an acceptor solution placed inside the lumen of the fiber. Based on the number of the phases used, HF-LPME is classified into two- or three-phases [18-20]. The main features of this technique include low cost, excellent clean-up efficiency and high enrichment factors. Whilst, the outstanding disadvantage of HF-LPME is the lack of selectivity due to the limited contribution of organic solvents sustained in the wall pores (two-phase mode) and sometimes also in the lumen (three-phase mode) of the hollow fibers [18-20]. Moreover, the HF-LPME has relatively long response time associated with the analyte diffusion process, resulting adverse effects on the method performance [21-23].

An approach to further improve the extraction efficiency, stability and selectivity of HF-LPME, reinforced hollow fiber solid/liquid phase microextraction (HF-SLPME) using 1-octanol as organic phase with different reinforced solid materials have been applied for determination of drugs in different biological fluids [24-32].

Multi walled carbon nanotubes (MWCNTs), a graphitic sheet rolled up into a cylindrical shape, are considered as prospective candidates in various preconcentration techniques [33-35]. Specifically, hexagonal arrays of carbon atoms in graphene surface of MWCNTs have a conjugated π-bond structure and strong interaction with certain analytes contained aromatic rings [36-38]. Therefore, they have been widely used for extraction of different aromatic-based compounds such as: poly-phenols [39], phthalate esters [40, 41], chlorophenols [42, 43] and diethylstilbestrol [25]. However, the lack of solubility and the difficult manipulation in organic solvents together with their weak affinity to the most polymer matrices have imposed great limitation to the use of MWCNTs [33, 44-46]. To solve these problems, functionalization of MWCNTs, by which carboxylic acid groups and hydroxyl groups could be added onto the surface of MWCNTs, is an effective process to help the MWCNTs dispersion in the target phases (organic solvent and polymer) [26, 47, 48]. Furthermore, the chemical functionalization of MWCNTs, broadens the potential for preparing new functional groups with interesting and promising applications in microextraction techniques [49, 50].

In this study, a new designed of functionalized MWCNTs reinforced hollow fiber solid-liquid phase microextraction (F-MWCNTs-HF-SLPME) device was developed to extract and preconcentrate of PHP from spiked human urine samples. The functionalized MWCNTs (F-MWCNTs) was used as a reinforcement material to enhance the stability of organic phase located in the lumen sealed device and also to facilitate the adsorption of PHP from the urine sample to the organic phase. Moreover, tumbling of the extraction device freely into the sample solution during the extraction will reduce the Nernst diffusion layer and improve both the rate and the extraction efficiency of the proposed method.

2. EXPERIMENTAL

2.1. Chemicals and Reagents

Phenazopyridine hydrochloride, diethyl ether, sodium dihydrogen phosphate (all purity grades > 98 %), and sodium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany). 1-Octanol, HPLC-grade organic solvent was obtained from Merck (Darmstadt, Germany). Multi-walled carbon nanotube (MWCNTs) with dimensions of 20- 40 nm outer diameter and 5-15 μm length was purchased from Shenzhen Nanotech Port Co. (China). The Q3/2 Accurel polypropylene hollow fiber membrane (600 μm i.d., 200 μm wall thickness, and 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany).

2.2. Preparation of Standard Solutions and Urine Samples

The stock solutions of PHP (1 mg mL⁻¹) were prepared in methanol and stored in brown flasks at 4°C. All the working solutions were freshly prepared by the appropriate dilution of the stock solutions with deionized water to obtain the desired concentrations.

Drug-free human urine samples were obtained from healthy volunteers. Urine standard solutions were prepared daily by using freshly drug-free urine with PHP stock solutions, followed by adjustment to pH 9.0 using 0.1M of NaOH solution.

2.3. Functionalization of MWCNTs

The functionalization of MWCNTs by oxidation using concentrated acid was performed as previously reported [51] with some modifications. 0.5g of MWCNTs was immersed in 100 mL of concentrated HNO₃ and ultrasonicated in a water bath for 24 h at room temperature. Then, the mixture was filtered and washed with deionized water until the pH reached 7.0. Afterward, the functionalized MWCNTs (F-MWCNTs) were dried at 70 °C and saved until used.

2.4. Preparation of F-MWCNTs-HF-SLPME Device

Polypropylene hollow fibers micro-tube (HF) was cut into small segments with a length of 2.5 cm and rinsed in acetone for 5 min to remove any possible impurities. The HF was then entirely immersed in a solution containing 30 mg L⁻¹ of F-MWCNTs in diethyl ether for 10 min under ultrasonica-
tion at room temperature. The purpose of this process is to allow the functionalized MWCNTs to co-deposit on the porous surface of HF, followed by a drying procedure at room temperature and maintained for 30 min, to ensure that all the diethyl ether has been evaporated. Then the F-MWCNTs-reinforced-HF micro-tube was filled manually with 1-octanol using a 1-mL micro syringe, after that the two ends of the micro-tube were heat-sealed by means of a hot soldering tool and the surface of the device was washed with ultrapure water to remove excess 1-octanol remained. Fig. (2) shows the Scanning Electron Microscopic (SEM) image of the F-MWCNTs-HF-SLPME device, which clearly represents the presence of this material in the pores of the composite HF.

2.5. Extraction Procedure of the Developed Method

A 10-mL aliquot of sample solution was adjusted to different pH values of sodium hydroxide solution and placed in a 10-mL of a glass beaker as shown in Fig. (2). The prepared F-MWCNTs-HF-SLPME device was introduced into the sample solution and stirred at different speeds. At the end of extraction, the device was removed and dried with a lint-free tissue then put into a HPLC-micro-vial contained organic solvent for desorption of the analyte via ultra-sonication. Afterwards, the HPLC-micro-vial was transferred to HPLC-DAD for analysis. Each device was employed only once to avoid any possibility of memory effect.

2.6. HPLC-DAD Instrumentation and Conditions

Analysis was performed using an HPLC Ultimate 3000 system (Dionex, Germering, Germany) consisted of a quaternary pump, an autosampler, a column heater and UV/DAD detector. Chromatographic separation was developed on an Inertsil ODS-3, C18 (150 mm×4.6 mm×5 μm) column (Merck Germany). A binary gradient elution system consisting of solvent A (methanol) and solvent B (phosphate buffer: 10 mmol/L, pH 6.5) is used as mobile phase.

Separation was carried out at room temperature on an Inertsil ODS-3, C18 (150 mm×4.6 mm×5 μm) reversed-phase column (Merck Germany). The gradient program was run from 85% B, decreased to 75% over a period of 5 min and then to 20% within 1-min using linear gradients. After 9 min the mobile B returned to the starting conditions 85% and the program was terminated at 10 min. The flow rate was maintained at 1.8 mL min⁻¹, total run time was set to 10 min. Detection was performed at 395 nm and injection volume was 20 μL with column temperature set to 32°C. Retention time (t_r) of PHP was at 7.20 min.

3. RESULTS AND DISCUSSION

3.1. Optimization of the F-MWCNTs-SLPME Conditions

In this study, the F-MWCNTs-HF-SLPME method followed by HPLC-DAD is used for the preconcentration and determination of PHP in human urine samples. The principle extraction mechanism of this method is based upon using the F-MWCNTs nanocomposite as solid-phase absorbent and 1-octanol act as the liquid-phase extractant. Therefore, the mass transfer of PHP involves both solid- and liquid-phase extractions. After dipping the F-MWCNTs-HF-SLPME device in the analyte sample solution, the PHP will adsorb on the surface of F-MWCNTs (already presented in the porous of HF) arises from two possible of electrostatic interactions. The PHP π-bonds (see Fig. 1) will have π-anion interaction with carboxylic groups and π-π interaction with conjugated π-carbon bond structure both located on the surface of F-MWCNTs. Afterward, the adsorbed PHP will diffuse through the membrane and will be trapped in 1-octanol organic phase that already fills the lumen of HF.

Several parameters affecting the extraction performance of developing method were studied and optimized including pH of sample solution, number of F-MWCNTs-HF-SLPME device, extraction time, stirring speed and desorption condition including type, volume of organic solvent and desorption time. All the determination steps were averaged from three replicate measurements.

Fig. (2). Schematic diagram of the F-MWCNTs-HF-SLPME technique.
3.1.1. Effect the pH of Sample Solution

To achieve the highest sorption efficiency based on the electrostatic interactions between the analyte and the F-MWCNTs, the analyte should be electrically neutral. Since PHP is a weak basic compound (with pKa 6.5) [52], the basicity of the sample solution should be differing from the pKa value of the analyte about 2 or 3 units to guarantee that most of the analyte exist mainly in neutral state [13]. Hence, the effect of pH values of sample solution was investigated ranging from 7 to 11 using 0.1 M of NaOH solution (Fig. 3). The extraction conditions were as follow: 0.5 μg L⁻¹ of PHP in 10 mL sample solution, 2 F-MWCNTs-HF-SLPME devices, 15 min extraction time stirring speed 300 rpm and 250 μL of methanol as desorption solvent for 5 min desorption time. It was found that the extraction efficiency increased as the pH increased from 7 to 9 and remained almost unchanged. Therefore, pH 9 was selected as the optimum pH of sample solution for further studies.

3.1.2. Effect the Number of Extraction Devices

The effect of the number of F-MWCNTs-HF-SLPME devices on the extraction efficiency of the developing method was also tested by using 10 mL of pH 9 sample solutions spiked at 0.3 μg L⁻¹ of PHP, time of extraction and desorption were 40 and 10 min, respectively and for other extraction conditions refer to section 3.1.1. As predicted, upon increasing the number of F-MWCNTs-HF-SLPME devices (from 1 to 4), the extraction efficiency was increased (Fig. 4). Nevertheless, no additional enhancement was detected when more than four devices were used. Consequently, four devices were used, as an optimum number, for the remaining studies.

3.1.3. Effect the Extraction Time

Another important parameter that needed adjustment is the time of extraction. This parameter was studied by monitoring the variation of extraction efficiency for the target analyte with time (varied from 10 to 60 min) under other optimized conditions. As shown in Fig. (5), the signal rose continuously up to 40 min and then remained constant, thus, 40 min was selected as the optimum extraction time for the successive experiments.

3.1.4. Effect the Stirring Speed

An appropriate stirring speed of the sample solution could accelerate the mass transfer process of analytes and increase the extraction efficiency. The effects of stirring speed on the extraction efficiency of the target compound was also investigated thoroughly at stirring speeds of 100-500 rpm. The stirring speed at 300 rpm provided the best extraction efficiency of PHP. Upon setting the agitation above 300 rpm the formation of bubbles was recognized, bubbles tend to adhere to the surface of the fiber and reduce the extraction efficiency. Thus, stirring at 300 rpm was chosen for further studies.

3.1.5. Effect the Desorption Condition

The desorption condition, including type of desorption solvent, volume of desorption solvent and desorption time were also evaluated. Acetonitrile, methanol and water were tested with ultra-sonication. Methanol with the lowest value of dielectric constant [53] gave the best capability to release the PHP from the solid/liquid extract and hence was adopted as the desorption solvent for the subsequent experiments.

Several volumes of methanol were also investigated. These volumes varied from 100 to 300 μL. Consequently, volumes lower than 250 μL of methanol were not sufficient to immerse all the F-MWCNTs-HF-SLPME devices during the ultrasonication, while, methanol volumes higher than 250 μL caused a decrease in the extraction efficiency resulting from the dilution of the analyte. Thus, 250 μL has been chosen to be used during the desorption process.
Moreover, the effect of sonication time with methanol (5–15 min) was investigated. It was found that 5 min was the optimum time for the next experiments.

Based on the above-described experiments, the optimal F-MWCNTs-HF-SLPME conditions for the PHP were selected as follows: 4 F-MWCNTs-HF-SLPME devices, pH 9 using NaOH as the medium for the 10 mL of sample solution. The extraction time was performed for 40 min, 300 rpm stirring speed with 250 μl of methanol as desorption solvent and a 5 min of desorption time.

3.2. Method Validation

In order to validate the feasibility of the proposed method, PHP was analyzed using spiked urine samples. Under optimized condition the analytical quality parameters: linearity, reproducibility, limit of detection (LOD), limit of quantification (LOQ) and accuracy were investigated. As shown in Fig. (6), no interfering peaks due to endogenous substances were observed at the retention time of the PHP. The linear dynamic range was obtained from 0.01 to 10 μg L⁻¹. The regression equation was \( y = 9.4202x + 0.6944 \) with a squared correlation coefficient (R²) of 0.9997. The relative standard deviations (RSD, n=5) for every concentration used were: 0.01 μg L⁻¹ (3.7%), 0.05 μg L⁻¹ (3.4%), 0.07 μg L⁻¹ (2.9%), 0.1 μg L⁻¹ (2.1%), 1 μg L⁻¹ (1.7%), 5 μg L⁻¹ (1.8%), 7 μg L⁻¹ (1.5%) and 10 μg L⁻¹ (0.9%). Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on three-times of standard deviation of the blank (3 Sb/m) and (10 Sb/m) respectively and the LOD/LOQ value was found to be 0.02/0.09 μg L⁻¹. The accuracy of the F-MWCNTs-HF-SLPME method was verified by means of recovery studies. The recovery was determined in urine samples spiked at three concentration levels: low (0.01 μg L⁻¹), medium (0.5 μg L⁻¹) and high (10 μg L⁻¹). Each sample was analyzed three times. The average recovery of the PHP using the developed method was 93.5% with the RSDs 2.7%.

Fig. (6). Chromatogram obtained from the F-MWCNTs-HF-SLPME of 10 mL of (A) a urine sample spiked with 0.1 μg L⁻¹ of PHP and (B) a blank urine sample.
3.4. Comparison of Method

The comparison of analytical performance data between the proposed method with other referenced techniques for the determination of PHP in urine samples is summarized in Table 1. The analytical performance of F-MWCNTs-HF-SLPME medium exhibits some advantages. The urine samples pretreatment for this method was based only on the adjustment of the pH, while for the other compared methods, several complicated steps were needed prior to the extraction procedure. Moreover, it was observed that in comparison with most of other listed methods, the present method consumes little organic solvent. Finally, in our proposed method, the most parameters such as LOD, RSD, and recovery are comparable with HF-LLLME-FIA and much better than other listed methods.

CONCLUDING REMARKS

The present study has demonstrated that the F-MWCNTs-HF-SLPME forwarded with HPLC-DAD may be successfully utilized for the determination of phenazopyridine in urine sample. In fact, the advantages of the F-MWCNTs-HF-SLPME including high efficient preconcentration and interference elimination, the method uses only a few microliters of an organic solvent (1-octanol). Therefore, this method could be considered as a green method for pharmaceutical analysis. Simplicity, good precision and lower LODs of this extraction technique process make it an attractive alternative to other referenced methods.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

REFERENCES


