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Ruthenium Nano Particles as an Efficient Probe Followed by Dispersive Liquid-Liquid Microextraction and Spectrofluorimetry for Determination of Gemfibrozil

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ABSTRACT

Ruthenium nano particles assisted dispersive liquid-liquid microextraction (DLLME) combined with spectrofluorimetry was applied to the extraction, pre-concentration and analysis of gemfibrozil. Microextraction solvent and disperser solvent containing nano particles are directly injected into an aqueous solution containing TC. After centrifuging, phase separation is performed by sedimenting the fine droplets of the microextraction solvent on the bottom of a test tube. The settled phase is transferred into a fluorometer for the determination of gemfibrozil at excitation/emission wavelengths of 375/438 nm. Under the optimized experimental conditions, the method provides a linear dynamic range of 0.2 to 100 ng mL^{-1}, a detection limit of 0.06 ng mL^{-1}, and a relative standard deviation of 3.0%. The method was successfully applied to pharmaceutical formulations and human urine. The results were validated by recovery test and by comparison with other methods, and were found to be highly satisfactory.

KEYWORDS: Gemfibrozil, Spectrofluorimetry, Dispersive liquid-liquid microextraction, Pharmaceutical, Urine

1.INTRODUCTION

Gemfibrozil, 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid, is a benzene derivative of valeric acid with lipophilic character and poorwater solubility.

Various analytical methods have been reported for the determination of gemfibrozil in both pharmaceutical preparations and biological samples. These methods include spectrophotometry [1-3], spectrofluorimetry [4-10], high performance liquid chromatography (HPLC) [11,12], capillary electrophoresis [13] and voltammetry [14]. However, some of the reported methods for the determination of gemfibrozil are time-consuming, tedious or insensitive. HPLC methods are widely combined with a pre- or post-column derivatization which takes too much time, and using toxic reagent is a common practice in these methods. In addition, the levels of gemfibrozil in some real samples are lower than detection limit of these techniques. Thus, the clinical investigations of gemfibrozil in pharmaceutical and biological samples require sensitive, simple and inexpensive analytical methods without time-consuming steps prior to analysis. However, most of these methods are relatively expensive and are not accurately reliable for the determination of trace amounts of gemfibrozil. Also, due to matrix effects and low concentrations of gemfibrozil in different samples, using separation and pre-concentration steps are still necessary and coupling of them with simple and less expensive determination techniques such as spectrofluorimetry is very attractive.

The use of separation and pre-concentration steps based on dispersive liquid-liquid microextraction (DLLME) offers conventional alternative to more conventional extraction systems and permits the design of extraction schemes that are simple, cheap and efficient. The principles, advantages and applications of DLLME have been well-established and identified in recent years. DLLME methodology has been successfully applied for pre-concentration and micoextraction of a wide range of organic compounds and metallic ions in different samples [15-18]. To the best of our knowledge, up until now, little attention has been paid to extraction, pre-concentration and determination of drugs based on DLLME. DLLME is a modified solvent extraction method and its acceptor-to-donor phase ratio is greatly reduced comparing with other extraction methods. Simplicity of operation, rapidity, low sample volume, low cost and high enrichment factor is some advantages of DLLME.

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Spectrofluorimetric methods are the most commonly used techniques and continue to enjoy wide popularity. The common availability of the instrumentation, the simplicity of procedures, sensitivity, speed, precision and accuracy of the technique still make spectrofluorimetric methods attractive. The analytical advantages of the application of fluorescence to gemfibrozil determination are its proper selectivity, sensitivity and wide dynamic range. In addition, DLLME shows high efficiency for quantitative extraction and operative simplicity[19-21].

In this paper, for the first time, a new combined methodology of Ruthenium nano particles assisted dispersive liquid-liquid microextraction (DLLME) and fluorimetric determination of gemfibrozil has been developed[21,22]. The results obtained showed that this methodology is very satisfactory for predicting the concentrations of the gemfibrozil in pharmaceutical dosage forms and human urine samples without tedious pre-treatment.

2.Experimental

2.1.Instrumentation

All fluorescence measurements were made using a Perkin-Elmer LS 50 spectrofluorimeter equipped with xenon discharge lamp, and quartz micro-cell with a path length of 10 mm and a volume of 55 µL. Instrument excitation and emission slits both were adjusted to 15 nm. A centrifuge from Hettich (Tuttlingen, Germany, www.hettichlab.com) was used for centrifuging. The pH-meter model 692 (Herisau, Switzerland, www.metrohm-ag.com) supplied with a glass-combined electrode and universal pH indicator (pH= 0-14) from Merck (Darmstadt, Germany, www.merck.de) were used for the pH measurements. An adjustable sampler (10-100 µL) was prepared from Eppendorf (Hamburg, Germany). A 1 mL syringe was prepared from Hamilton (Reno, NV, USA, www.hamiltoncompany.com).

2.2.Assay procedure for gemfibrozil tablets

Ten gemfibrozil tablets, labeled as containing 250 mg gemfibrozil each, were weighed and the average mass per tablet was determined. An amount of the powder equivalent to 5 mg of gemfibrozil was accurately weighed and dissolved in ultra pure water in a 100 mL calibrated flask by sonication The solution was filtered into a 100 mL calibrated flask through Whatman no.42 filter paper and diluted to an appropriate volume with the same solvent to achieve a final concentration of 50 µg mL^{-1}.

2.3.Spiked human urine samples

Urine samples, obtained from healthy volunteers, were collected and mixed. Aliquot of 10 mL from this mixture were placed in graduated centrifuge tubes. These solutions were centrifuged for 5 min at 6000 rpm and 2 mL of supernatants were transferred into new test tubes and stored frozen until assays. Aliquots of centrifuged human urine samples (each of 200 µL) were spiked with different amounts of gemfibrozil (5, 35, 50 and 75 ng mL^{-1}). Each of these samples was put in a centrifuged tube and then the analysis was followed up as indicated in the general procedure.

2.4.General procedure

For Ruthenium nano particles assisted dispersive liquid-liquid microextraction (DLLME)under optimum conditions, 10.0 mL of sample solution containing the analyte (pH = 13) was transferred into a glass test tube with conic bottom. After a few seconds, 0.50 mL of acetone (disperser solvent) containing 122 µL of chloroform (microextraction solvent) was injected rapidly into the sample solution by using a 1 mL syringe. A cloudy solution (water, acetone and chloroform) was formed in a test tube. Then nano particles were added. The mixture was then centrifuged for 2 min at 4000 rpm. After this process the dispersed fine droplets of chloroform were sedimented at the bottom of conical test tube (60 µL) and then this sedimented phase was removed using a sampler and injected into a 55 µL micro-cell. Subsequently, the micro-cell was located in spectrofluorimeter to obtain related spectra. The fluorescence intensity was measured at 438 nm with the excitation wavelength set at 375 nm.

2.5.Reference voltammetric method

A voltammetric procedure was applied as the reference method [17]. In this procedure, ten millilitres of 0.01 mol L^{-1} supporting electrolyte were transferred into the cell and deaerated by passing nitrogen through for 10-16 min. The working electrode was a mercury drop electrode. The reference electrode (to which all potentials are referred) and the counter electrode were Ag/AgCl/KCl (3 mol L^{-1}) and platinum wire, respectively. An accumulation potential of -1.4 V and a scan rate of 100 mV s^{-1} were used. After the accumulation step and a further 15 s (equilibrium time), the voltammogram was recorded. The gemfibrozil solution was introduced using an automatic pipetter. The mixture was stirred while purging

with nitrogen, and then the deposition and stripping step were followed. All the results were obtained at room temperature with a nitrogen atmosphere maintained over the solution surface.

3.RESULTS AND DISCUSSION

In this research, DLLME was combined with spectrofluorimetry for the first time and gemfibrozil was chosen as an example analyte to investigate the feasibility of this joining. Gemfibrozil reacts with ferricyanide in alkaline medium and forms gemfibrozil , which is subsequently trapped in a low volume of microextraction solvent and separated from the aqueous phase or biological matrix. The excitation and emission spectra of gemfibrozil are with maxima at 375 ± 4 and 438 ± 4 nm, respectively. The influence of different factors affecting extraction conditions, such as kind of microextraction and disperser solvent and their volume, pH, ionic strength and extraction time were studied and optimized to obtain a compromise between pre-concentration factor, sensitivity, simplicity and reproducibility. Pre-concentration factor (PF) and percent extraction recovery (ER%) as analytical responses were calculated based on the following equations:

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

$$ER\% = C_{sed} \times V_{sed} / C_0 \times V_{aq} \times 100$$

where Csed and C0 are concentration of the analyte in the sedimented phase and initial concentration of the analyte in the aqueous sample, respectively. Vsed and Vaq are the volume of the sedimented phase and volume of the aqueous sample, respectively. Csed, for each microextraction solvent, was calculated on the calibration graph which obtained from conventional LLE combined with spectrofluorimetry.

Selection of microextraction solvent

The selection of an appropriate microextraction solvent is critical for the DLLME process. The microextraction solvent has to meet some requirements: it should have a higher density than water, a low solubility in water, and high extraction efficiency of the compound of interest; it also should have no interference with the analyte peak when directly transferred into a micro-cell of fluorimeter for analysis. In the present study, chloroform (density, 1.48 g mL^{-1}), carbon tetrachloride (density, 1.58 g mL^{-1}) and carbon disulfide (density, 1.26 g mL^{-1}) were selected as microextration solvents. ER% using chloroform, carbon tetrachloride and carbon disulfide were 69.2%, 42.7% and 36.8% respectively. The results revealed that chloroform has the highest extraction recovery in comparison with the other tested solvents. It is probably because of higher solubility of TC in chloroform in comparison with carbon tetrachloride and carbon disulfide. Therefore, chloroform was selected as the optimum solvent.

3.1.Selection of disperser solvent

The miscibility of the disperser solvent in the organic phase (microextraction solvent) and the aqueous phase (sample solution) is the main point for the selection of the disperser solvent in the present pre-concentration technique. Acetone, acetonitrile and methanol, which show this ability, were chosen as disperser solvents. A series of sample solutions were studied by the injection of 0.50 mL of each disperser solvent containing 122 µL chloroform (as microextraction solvent) into the test tube including sample solution. Considering the sedimented phase volume, it was found that with combination of chloroform-acetonitrile, the sedimented phase volume was very higher than 60 µL and the cloudy state was not formed well, whereas in the case of chloroform-methanol, and chloroform-acetone, the sedimented volume was about 60 µL. Thus, acetone and methanol could be selected as disperser solvents for further studies. Further experiments revealed that the PF in the presence of acetone and methanol were 115.4 and 96.2, respectively. According to the results, acetone has the higher per-concentration factor, lower toxicity and lower cost in comparison with methanol. Therefore, acetone was used for further studies.

3.2.Influence of microextraction solvent volume

The recovery of about 60 µL of the sedimented phase after DLLME is necessary because of the use of a 55 µL micro-cell in all experiments. Also, it is necessary to add an excess amount of the microextraction solvent due to its solubility in water. In order to evaluate the effect of extraction volume on the extraction efficiency, additional experiments were done using solutions containing different volumes of chloroform (122, 132, 142 and 152 µL) and fixed volume of acetone (0.50 mL). With increasing of the microextraction solvent volume from 122 to 152 µL, the volume of the sedimented phase increased (60-98 µL). Using less than 122 µL volume of chloroform decreased the volume of the

sedimented phase to less than 60 µL, thus, removing the sedimented phase for injection into a 55 µL micro-cell of fluorimeter would be too difficult and accompanies systematic error.

The variation of fluorescence intensity versus volume of the microextraction solvent (chloroform) was investigated. These results show that signal intensity decreases as the volume of chloroform increases due to increase in the sedimented phase volume. So, 122 µL of chloroform was selected in order to achieve a compromise between simplicity, sensitivity and reproducibility.

3.3.Influence of disperser solvent volume

Volume of the dispersive solvent is one of the important factors to be considered. At lower volumes of the disperser, tiny droplet formation may not be effective thereby lowering the extraction efficiency. At higher volumes of the dispersive solvent, the solubility of TC in aqueous solution increases; thus, lowering the partition of TC into chloroform leading to a decrease in efficiency. The variation of the acetone (as disperser solvent) causes changes in the volume of the sedimented phase. To prevent this problem, and in order to acquire a constant volume of the sedimented phase, the volumes of the acetone and chloroform were changed, simultaneously. Other experimental conditions were kept constant and included the use of different acetone volumes, 0.25, 0.50, 1.0 and 2.0 containing 100, 122, 140, 169 µL chloroform, respectively. In this step, the volume of the sedimented phase was constant (60 ± 3 µL). The influence of disperser solvent (acetone) volume on the spectrofluorimetic responses was studied. The results indicate that an increase in the volume of acetone up to 0.50 mL leads to an increase in the fluorescence intensity and then to a decrease. It seems that, in the lower volumes of acetone, a cloudy state is not formed well, thereby, the pre-concentration factor is low. In higher volumes of acetone, solubility of TC in aqueous solution increases. Therefore, the extraction efficiency decreases due to the decrease of distribution coefficient. A 0.50 mL of acetone was chosen as optimum value.

3.4.Influence of pH

Effect of pH on the oxidation efficiency of gemfibrozil was investigated in the range of 2-13 using hydrochloric acid and sodium hydroxide. Fluorescence intensity increases linearly with alkalinity and reaches a constant value from 12. The speed of the oxidation increased as pH attained more basic values. In addition, these results indicate that in the pH rang of 2-6 gemfibrozil is not oxidized to TC by ferricyanide. Hence, in all later experiments, a pH of 13 was chosen to obtain a compromise between sensitivity, oxidation efficiency, speed and reproducibility. A series of supplemental experiments were carried out by using different buffer systems. In this study, pH of a series of solutions containing analyte and other reagents was adjusted in 13 by using OH- or different buffer systems and solutions were subjected to the DLLME and spectrofluorimetric determination. The results showed that higher analytical signals were obtained by using OH- as buffering agent. So, the pH adjustment was not carried out by using buffer systems and addition of proper amount of OH- is sufficient for adjustment of pH and achievement of higher analytical signal.

3.5.Influence of ionic strength

The impact of ionic strength on extraction efficiency and subsequent measurement was evaluated by adding different amounts of NaCl (0-5% (w/v)). Other experimental conditions were kept constant. Based on the results obtained in this study salt addition has no significant and benefic effect on the extraction efficiency. On the other hand, the enrichment factor is nearly constant by increasing the amount of sodium chloride. Thus, this electrolyte was not used through the rest of the work.

3.6.Influence of the extraction time

Extraction time is one of the major parameters affecting the extraction efficiency, especially in microextraction methods such as SPME and LPME. In the DLLME method, the extraction time is defined as an interval between the injection of the mixture of disperser solvent (aceton) and the microextraction solvent (chloroform), and starting centrifuge. The dependence of extraction efficiency upon extraction time was investigated within a range of 5-300 sec. Based on the results obtained in this study signal variations versus extraction time were not significant. It was revealed that after formation of cloudy solution, the surface area between extraction and aqueous phase was infinitely large. Therefore, the transfer of TC from aqueous phase to microextraction solvent was very fast. Subsequently, the equilibrium state was obtained quickly and the extraction time was very short. In this method, the most time-consuming step was the centrifuging of the sample solution in the extraction procedure, which was about 2 min. Thus, this method is very fast and this is the most distinct advantage of the DLLME technique.

3.7.Excitation and emission spectra of TC

It is well known that a great majority of the organic compounds which exhibit fluorescence possess cyclic, conjugated structures involved π-electron system. the maximum excitation and emission wavelength of TC treated as in the general procedure were 375 and 438 nm. As it can be ascertained, the excitation and emission of the blank of reagent has no effect on the determination of gemfibrozil. So, the mentioned wavelengths were selected as excitation and emission conditions, respectively. Optimum experimental conditions of DLLME-spectrfluorimetry for the determination of gemfibrozil are shown in Table 1.

4.Application

4.1.Analytical figures of merit

Under the optimum experimental conditions, the relationship between the analytical signal and concentration was studied over the range 0.01 to 500 ng mL^{-1} and found to be linear from 0.2 to 100 ng mL^{-1}. The corresponding fitted equation was I = 84.5 + 9.109 C with r_2 = 0.9991, where I is the fluorescence intensity at 438 ± 4 nm and C is the gemfibrozil concentration in ng mL^{-1}. The limit of detection was determined by using the criterion, LOD = ksbl/m, where k is a factor (=3), sbl is the standard deviation of the blank measurements and m is the calibration slope. The value thus found was 0.06 ng mL^{-1}. The repeatability of the system was determined from seven consecutive insertions of a 50 ng mL^{-1} standard solution of gemfibrozil; the relative standard deviation (R.S.D.) thus obtained was 3.0%.

Initially, the accuracy of the proposed method was evaluated by means of recovery experiences. For this purpose, different amounts of authentic gemfibrozil were added to the diluted sample solutions, obtained from gemfibrozil tablets, and subsequently assayed by the developed procedure. The results of this recovery study are shown in Table 3 of Electronic Supplementary Material. As can be ascertained, in all cases, the quantitative recoveries (91.4-103.5%) were obtained.

4.2.Interference study

In order to assess the possible analytical applications of the proposed method, the effect of concomitant species on the determination of gemfibrozil in real samples was studied. Samples containing a fixed concentration of gemfibrozil (50 ng mL^{-1}) and various excess amounts of the foreign species were subjected to the proposed method. The study was focused on some common drugs, excipients and some compounds abundant in human urine. The tolerance ratio of each foreign substance was taken as the largest amount yielding an error in the determination of the analyte not exceeding 5%.

4.3.Analysis of gemfibrozil in pharmaceutical preparation

The developed procedure was applied to the determination of gemfibrozil in the commercial pharmaceutical preparations. The sample solutions, obtained from gemfibrozil tablets, were treated as mentioned in the general procedure to calculate the amounts of gemfibrozil in the samples using simple calibration line. Table 2 presents results obtained by applying the present combined methodology and those obtained by a reported voltammetric method [17]. These results indicate the accuracy of the proposed method, and its viability for the analysis of gemfibrozil in this type of samples.

4.4.Determination of gemfibrozil in spiked human urine sample

In Fig. 1, spectra of urine sample with and without DLLME were compared, demonstrating the efficacy of the recommended method to eliminate interference from urine, which is a highly fluorescent matrix. Regarded to confirm the accuracy of this method, a calibration curve using spiked urine sample was made. The working interval of 5 to 100 ng mL^{-1} was chosen for estimating value in actual samples. Aliquots of 200 µL urine samples were spiked with four different concentrations of gemfibrozil at 5, 35, 50 and 75 ng mL^{-1} and recovery experiments were conducted for these samples as well. The results presented in Table 3 show satisfactory recovery data demonstrating the feasibility of applying the developed methodology in a complex biological matrix. An additional advantage of the presented combined methodology is that the calibration curve obtained from spiked urine sample show a good correlation compared to that for aqueous standard solutions. Thus, the determination of gemfibrozil in urine could be made by direct comparison with aqueous standard solution, at the same instrumental conditions. In addition, it is avoided the need of employing a high performance separation instrumental for the treatment of urine previous measurement, which is most cases are necessary for eliminating interferences species.

4.5.Comparison with other works

A comparison of the main analytical characteristics (i.e., LOD, LR and RSD) of the proposed DLLME-spectrofluorimetry method for the determination of gemfibrozil with those of some of the best previously reported methods for this purpose is confirmed. As can be seen, LOD of the proposed DLLME-spectrofluorimetry method, with a sample volume of only 10.0 mL, is significantly improved over all the previously proposed methods. In addition, the extraction time is very short, which indicates the fact that DLLME is a very sensitive, rapid and reproducible technique that can be used for the pre-concentration and determination of gemfibrozil from real samples.

5.Conclusion

The Ruthenium nano particles assisted dispersive liquid-liquid microextraction (DLLME)-spectrofluorimetric method developed in this work permits the fast, accurate and reliable determination of gemfibrozil in commercial pharmaceutical preparations and human urine. The accuracy of the new proposed method has been validated by performing a recovery test, as the result was compared with a voltammetric method. In addition, the proposed method improves sensitivity through the pre-concentration step, and it represents a good approach in the area of pharmaceutical and urine monitoring with low operation cost and simplicity of instrumentation.

Table & Figure Captions

Fig. 1 Urine sample spectra with and without DLLME procedure; λex =375 nm, slit width= 15 nm; λem =438 nm, slit width 15 nm. (a) Spectrum of gemfibrozil (50 ng mL^{-1}) as gemfibrozil in urine treated by DLLME; (b) spectrum of urine alone; (c) spectrum of urine alone treated by DLLME.

Table 1. Optimum experimental conditions of the DLLME-spectrofluorimetry method.

Parameter	Value
Microextraction solvent (chloroform) volume	122 µL
Disperser solvent (acetone) volume	0.50 mL
Ferricyanide concentration (volume; 1 mL)	0.01 mol L^{-1}
Sample pH	13
Ionic strength	NEa
Extraction time	<3
Excitation wavelength	375 ± 4 nm
Emission wavelength	438 ± 4 nm
Slit width b	15 nm

a No effect.
b For both excitation and emission slits.

Table 2. Results of analysis of gemfibrozil tablets by the proposed procedure and by a reported voltammetric method [17].

Claimed (mg/tablet)	Proposed method (mg)a	Reported method (mg)a	Error (%) b	Error (%) c
150	152.24 (±1.27)	154.33 (±1.01)	+1.49	-1.35
250	246.63 (±0.58)	256.15 (±0.84)	-1.34	-3.71

a Values in parenthesis give the standard deviation based on three replicates.
b Error against the declared value.
c Error against the reported method.

Table 3. Results of recoveries of spiked urine samples.

Sample	Gemfibrozil added (ng mL^{-1})	Gemfibrozil found (ng mL^{-1})	Recovery (%)a	RSD (%)a
urine	5	4.7	94.0	±5.14
	35	31.0	88.6	±9.23
	50	51.0	102.0	±3.88
	75	76.2	101.6	±3.50

a Average of three independent measurements.

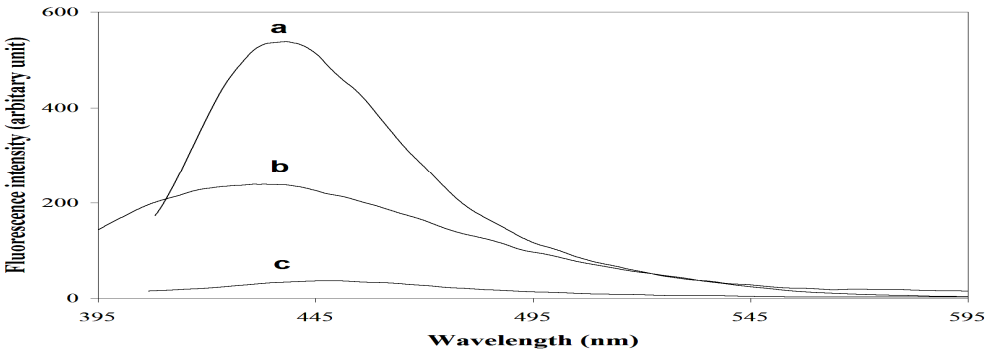

Fig.1. Urine sample spectra with and without DLLME procedure; λex= 375 nm, slit width= 15 nm; λem =438 nm, slit width 15 nm. (a) Spectrum of gemfibrozil (50 ng mL^{-1}) as gemfibrozil in urine treated by DLLME; (b) spectrum of urine alone; (c) spectrum of urine alone treated by DLLME.

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