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Determination of Trace Amounts of Risperidone in Human Urine Sample by Hollow Fiber Liquid-Phase Microextraction Combined with High Performance Liquid Chromatography

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Risperidone is an antipsychotic drug mainly used to treat schizophrenia. In this paper, for the first time a three-phase hollow ûber liquid-phase microextraction (HF-LPME) method combined with high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was presented to detection and quantification of risperidone in urine samples. Different factors affecting the HF-LPME extraction recovery were studied and optimized. Under optimal extraction condition, risperidone could be determined within the linearity range with a good correlation coefficient ($r^2 > 0.9980$). The limit of detection for risperidone was 0.2 ng mL^{-1}, and the intra- and inter-day relative standard deviations were no more than 1.2% and 3.2%, respectively. This procedure afforded a simple, sensitive and inexpensive method with a high extraction efficiency for determination of risperidone. Finally, it was applied to determination and quantification of risperidone in human urine samples.

Key words: Risperidone; High performance liquid chromatography; Hollow ûber based liquid phase microextraction; Microextraction.

Risperidone is an atypical antipsychotic, approved for the treatment of schizophrenia and for schizoaffective and bipolar disorders[1, 2]. The chemical structure, IUPAC name, the octanol-water distribution coefficient, and acidity constant of the target drug are classified in Table 1[3]. Risperidone belongs to a class of drugs called atypical antipsychotics. Risperidone is used to treat the manifestations of psychotic disorders. It appears to produce a significant improvement in both the positive and negative symptoms of schizophrenia. Risperidone is a selective monoaminergic antagonist with a strong affinity for serotonin type 2 (5-HT_2) receptors and a slightly weaker affinity for dopamine type 2 (D_2) receptors [4, 5]. The antipsychotic activity of risperidone may be mediated through antagonism at a combination of these receptor sites, particularly through blockade of cortical serotonin receptors and limbic dopamine systems. It works by helping to restitute the balance of certain natural substances in the brain. The exact mechanism of action of risperidone is not known, but, like other anti-psychotics, it is believed that risperidone affects the way the brain works by interfering with communication among the brain's nerves. Nerves communicate with each other by

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making and releasing chemicals called neurotransmitters. The attachment of the neurotransmitters either stimulates or inhibits the function of the nearby nerves. Risperidone blocks several of the receptors on nerves including dopamine and serotonin adrenergic receptors. It is believed that many psychotic sicknesses are caused by unusual communication among nerves in the brain and that by change communication through neurotransmitters, risperidone can alter the psychotic state[6-8]. People who take risperidone may suffer from a number of side effects, such as movement disorders, diabetes, pituitary tumors and heart problems that can lead to death[9-12]. Thus, it is necessary to introduce new methods that improve detection and measurement of risperidone in biological samples.

Numerous papers have been published on the analysis of risperidone in different matrices. Most of them concern with reverse phase high-performance liquid chromatography (HPLC)[13-18], HPLC with electrochemical detection[19, 20], Liquid chromatography–mass spectrometry[21-25], capillary zone electrophoresis[26], spectroscopic[27-29], and electrochemistry techniques[29-31].

Today, there is a constant need for the development of faster and more selective sample clean-up procedures. To best of our knowledge, microextraction technique has not been reported for extraction and preconcentration of risperidone from biological fluids. The aim of this study is the development of a sensitive, selective and fast analytical method, which allows the analysis of risperidone in biological fluids. In this work, for the first time, three phase hollow ûber based liquid phase microextraction (HF-LPME) technique followed by HPLC with ultraviolet (UV) detection was optimized and validated for detection and determination of risperidone in biological samples.

The concept of HF-LPME was developed by Pedersen-Bjergaard and Rasmussen in 1999[32]. In HF-LPME, target analytes are extracted from an aqueous sample, into the organic membrane solvent immobilized as a SLM in hollow fiber wall pores, and into the acceptor solution placed inside the lumen of the hollow fiber[33]. Today, HF-LPME is carried out in two modes: First, in two-phase HF-LPME, both the organic membrane solvent immobilized in the ûber pores and the acceptor phase are same[34-36]. Second, in three-phase HF-LPME, the analytes are extracted from the aqueous sample solution into the organic membrane solvent, and then into the aqueous acceptor phase inside the lumen[37-39]. Two-phase HF-LPME was performed in direct immersion and headspace extraction modes, whereas three-phase HF-LPME was performed only in direct immersion extraction mode[33]. Compared with two-phase HF-LPME, three-phase extraction has a much cleaner extraction .

The aim of this work was to develop a one-step method for extraction, preconcentration and sample clean up for the analysis of risperidone. To optimize the conditions of three phases HF-LPME, all effective parameters, including the organic membrane solvent, sample solution and acceptor phase pH, extraction time, stirring rate and salt addition effect, were optimized to the determination of the target analyte in urine samples.

EXPERIMENTAL

Chemical, reagents and standard solutions

Risperidone standard, kindly provided by Drug and Food Administration (Tehran, Iran). 1-Octanol, *dodecane*, isobutyl*methyl ketone*, and n-heptane were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile (HPLC grade) were from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide, sodium chloride, and orthophosphoric acid were obtained from Sigma–Aldrich. All of the chemicals used were of analytical-reagent grades. Ultrapure water was obtained by means of a MilliQ apparatus by Millipore (Madrid, Spain).

PPQ3/2 polypropylene hollow fiber was used in the extraction process from Membrana (Wuppertal, Germany) with the inner diameter of 0.6 mm, wall thickness of 200 µm, and pore size of 0.2 µm.

A 1000 mg L^{-1} stock solution of risperidone was prepared in methanol. The stock solutions were stable for at least two months when stored at -4 °C. Working solutions were prepared every day by diluting the stock solutions in pure water.

Apparatus and chromatographic conditions

A Younglin YL9100 HPLC (Seoul, Korea) equipped with a Quaternary9110 HPLC pump (Seoul, Korea), a 4-channel mixing valve with a 20

µL sample loop, YL9101 vacuum degasser and a YL 9120 UV-Vis detector was used for Separation and detection of the target analyte. Chromatographic data were recorded and analyzed using Younglin Auto Chro 3000 software. The separations were carried out on an ODS-3 column (150 mm × 4.6 mm, with particle size of 5 µm) from MZ-Analysentechnik (Mainz, Germany).The mobile phase consisted of 20 mM phosphate buffer, methanol, and acetonitrile (65:15:20), under isocratic condition. The flow rate of the mobile phase was set at 1.2 mL min^{-1}. Total analysis time was 8 min.The injection volume was 20 µL for all of the samples and detection wavelength was 276 nm.

Sample solutions were stirred using a MR Hei-standard magnetic stirrer from Heidolph (Schwabach, Germany). Sample solution and acceptor phase pH were adjusted by means of GPHR 1400 digital pH meter from Greisinger (Regenstauf, Germany).

HF-LPME extraction procedure

A new 8.5 cm length of hollow ûber was cut and washed with acetone in an ultrasonic bath for 10 min and dried at room temperature. Then, 50ìL Hamilton syringe stuffed full of acceptor solution and the tip of the Hamilton syringe was inserted into the lumen of the hollow fiber from one end. The fiber was then dipped in the organic membrane solvent (1-octanol) for 10 s to impregnate the pores of the hollow fiber wall, and then inserted the hollow fiber into the water for 3 s to wash the excess organic solvent from the hollow fiber surface. Then, the acceptor solution from the Hamilton syringe was filled into the lumen of the hollow fiber. First, 30 ìL of acceptor solution was flushed out of the fiber in order to remove any organic solvent remaining inside the lumen of hollow fiber, and then 20 µL acceptor solution was remained in the lumen and the lower end of the hollow fiber was mechanically sealed by a piece of aluminum foil. For each experiment, 15 mL of sample solution was filled into a 20 mL sample vial. The fiber was dipped into the sample solution and extraction was performed for a 40 min. During extraction, the solution was stirred at 750 rpm. After extraction, 20 ìL acceptor phase was collected into the micro-vial by syringe and finally, acceptor solution was injected into the HPLC instrument for analysis.

Biological sample analysis

Urine samples were collected from healthy young volunteer. The samples were stored at "4ÚC until required. The frozen urine sample was thawed and shaken at room temperature before use.

Calculation of preconcentration factor, and relative recovery

The analyte preconcentration factor (*PF*) is calculated by the following formula:

$$PF = \frac{C_{f,a}}{C_{i,s}} \quad ...(1)$$

where $C_{s,\ final}$ is the final analyte concentration in the acceptor phase, and $C_{o,\ initial}$ is the initial analyte concentration within the sample. Relative recovery (*RR*) was calculated by the following equation:

$$RR = \frac{C_{found} - C_{real}}{C_{added}} \times 100 \quad ...(4)$$

where C_{found}, C_{real}, and C_{added} are the concentrations (µg L^{-1}) of the analyts after addition of known amounts of the standard into the real sample, the concentration of analyte in real sample, and the concentration of known amounts of the standard which was spiked into the real sample, respectively.

RESULTS AND DISCUSSION

Selection of the organic solvent

To find an ideal extraction condition in HF-LPME, selection of an organic solvent is necessary for enrichment of the analyte. The selected organic membrane solvent should have a good tendency for the analyte, be immiscible with donor and acceptor phases, be compatible with the hollow fiber nature to be immobilized in the pores of hollow fiber wall, and have a low volatility to avoid evaporation during the extraction process [40]. In this work, different organic solvent such as 1-Octanol, *dodecane*, isobutyl*methyl ketone*, and n-heptane were compared for the impregnation of the ûber pores. As shown in Fig. 2A, 1-octanol showed the highest extraction recovery for risperidone extraction. Thus, 1-octanol was used in further experiments.

Optimization of the pH in the donor and acceptor phases

The pH of both the donor and acceptor

phases is a very important parameter for extraction of target analyte. It can influence the transfer of analyte from the donor sample solution to the organic membrane solvent, and then to the acceptor phase to obtain high extraction efficiency.

In this work, the donor sample solution was made alkaline to ensure that the risperidone were in a molecular form in order to increase their transfer from the sample solution into the organic phase. For this purpose, donor sample pH was investigated in the basic pH from 9 to 11. As shown in Fig. 2B, the highest extraction efficiency for risperidone was obtained using pH=10. Therefore, pH=10 was chosen for future experiments.

In current work, the acceptor phase was made acidic to ensure that the risperidone were in a ionized form to increase their transfer from organic phase into the aqueous acceptor phase. For determination of the composition of acceptor phase, pH of acceptor phase was studied from two to four. As shown in Fig. 2C, the highest extraction efficiency was obtained at pH=3.0. Therefore, the optimum pH of the acceptor phase was selected as 3.0.

Effect of stirring speed

Improvement of the extraction efficiency with stirring speed was evaluated. As a result shown in Fig. 2D, extraction efficiency was improved by the increasing stirring speed up to 750 rpm. In lower stirring speed, increasing stirring speed, dramatically accelerated extraction and shorten the time to reach the extraction equilibrium. At higher stirring speed, extraction efficiency decreased because the organic phase was removed from the ûber pores.

Effect of salt addition to the sample solution

In this work, the effect of salt added to extraction recovery of target analyte was examined by the addition of different concentration levels (0–30%, m/v) of NaCl to sample solution and result is shown in Fig. 3A. The aqueous solution viscosity would increase by addition of salt, which lead to difficult mass transfer and decrease extraction efficiency. The extraction efficiency, decreased when the NaCl concentration was increased from 0 to 10% (m/v). Therefore, extraction without salt addition was chosen as best condition, and used in the next experiments.

Effect of extraction time

The mass transfer is a time-dependent process. Mass transfer has two parts in HF-LPME: First, partitioning of the target analytes from donor

Table 1. Chemical structures, pK_a and log$P$ of risperidone.

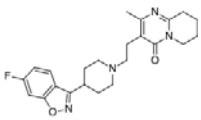
Name	Chemical Structure	IUPAC name	pK_a	log$P$
Risperidone		4-[2-[4-(6-fluorobenzo[d]isoxazol-3-yl)-1-piperidyl]ethyl]-3-methyl- 2, 6-diazabicyclo[4.4.0]deca-1,3-dien-5-one	8.76	2.63

Table 2. Figures of merit of HF-LPME in distilled water sample

LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Linearity (ng mL^{-1})	R^2	PF[a]	RSD% [b] Within day	Between day
0.2	1.0	1.0-2000.0	0.9980	157	1.2	3.2

[a] Drugs were present at 1.0 mg L^{-1}.[b] Within day and between day RSDs% were obtained by five replications.

Table 3. Determination of risperidone in urine sample

Sample	C_{real} (mg mL^{-1})	C_{added} (mg mL^{-1})	C_{found}(mg mL^{-1})	RSD% (n = 5)	RR%
Urine	nd[a]	0.2	0.173	5.5	87

[a] Not detected

phase to organic solvent and second, diffusion of the analytes through the impregnated solvent to the acceptor phase. Hence, the extraction recovery depends on both the transfer of the analyte from the donor phase to the organic membrane solvent and then the transfer from the organic solvent to the acceptor phase[41]. Thus, the effect of the extraction time was studied from 20 to 55 min. The result in Fig. 3B show that the extraction recovery increased rapidly by the increasing of the extraction time up to 40 min. Further increasing during the extraction time to 55 min, lead to dramatically decrease in the extraction recovery due to organic membrane unstability in sample solution. therefore, 40 min was chosen as the best extraction time in the next experiments.

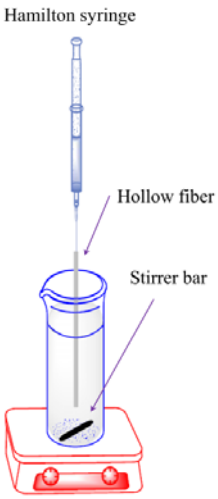


Fig. 1. Schematic diagram of proposed HF-LPME setup.

Inûuence of temperature

The effect of temperature on the extraction recovery of risperidone by HF-LLLME was examined over a temperature range of 20–60 °C and result is shown in Fig. 3C. Increasing of temperature from 20 to 40 °C increase extraction recovery. Increasing extraction temperature accelerates the mass transfer rates of analytes and increase extraction recovery. But when the extraction temperature increased higher than 40 °C, tangible change in extraction recovery was not observed. Therefore, 40 ºC was selected as the optimum temperature in this work and used in next experiments.

Method validation

Under the optimized conditions, a series of experiments were carried out to determine the linear ranges, repeatability, preconcentration factor, and LODs of this method that listed in Table 2. The calibration curves were linear in the range of 1.0–2000 µg L^{-1} with coefûcient of determination (r^2) more than 0.9980. The relative standard deviations (RSD %) for extraction and determination of risperidone were less than 1.2% and 3.2% for intraday and interday experiment, respectively. LODs less than 0.2 ng L^{-1} was observed for risperidone. PF values 157-fold were viewed for the extraction of risperidone.

Application to real samples

The optimized HF-LPME method was applied to the determination of risperidone from untreated urine sample. In order to reduce matrix effects, calibration curves were plotted in drug free urine sample.

Extraction from human urine sample

Drug-free human urine was spiked with

Table 4. Comparison of the HF-LPME with other analytical techniques for determination of risperidone.

Analytical method	Sample preparation method	Sample	LOD (ng mL-1)	Linearity (ng mL-1)	RSD%	Ref.
HPLC	HF-LPME	Urine	0.2	1.0-1000.0	3.2	This work
HPLC-MS-MS	SPME	Water	50.0	50.0-3750.0	<10	[1]
HPLC-EC	SPE	Plasma	0.5	1.0-100.0	<13.9	[42]
HPLC-UV	LLE	Plasma	5.0	10.0-160.0	<8	[19]
LC-Coulmetric	MEPS	Plasma/urine	0.5	0.5-50	<5.8	[43]
HPLC-UV	MEPS	Plasma/urine	0.07	2.0-200.0	<4.4	[2]
LC-MS-MS	SPE	Plasma	0.1	0.1-250.0	<4.5	[44]
HPLC-UV	LLE	Plasma	1.5	2.0-100.0	<15	[45]
LC-MS	LLE	Plasma	0.1	0.2-24.0	<15	[46]

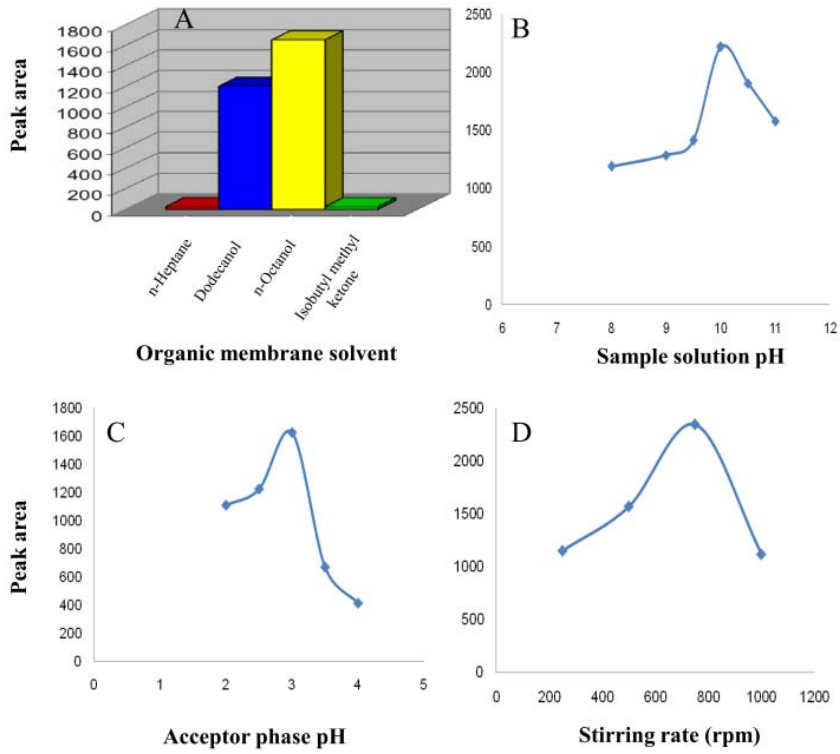


Fig. 2. Optimization of (A) organic membrane solvent, (B) sample solution pH, (C) acceptor phase pH and (D) stirring rate for extraction of risperidone

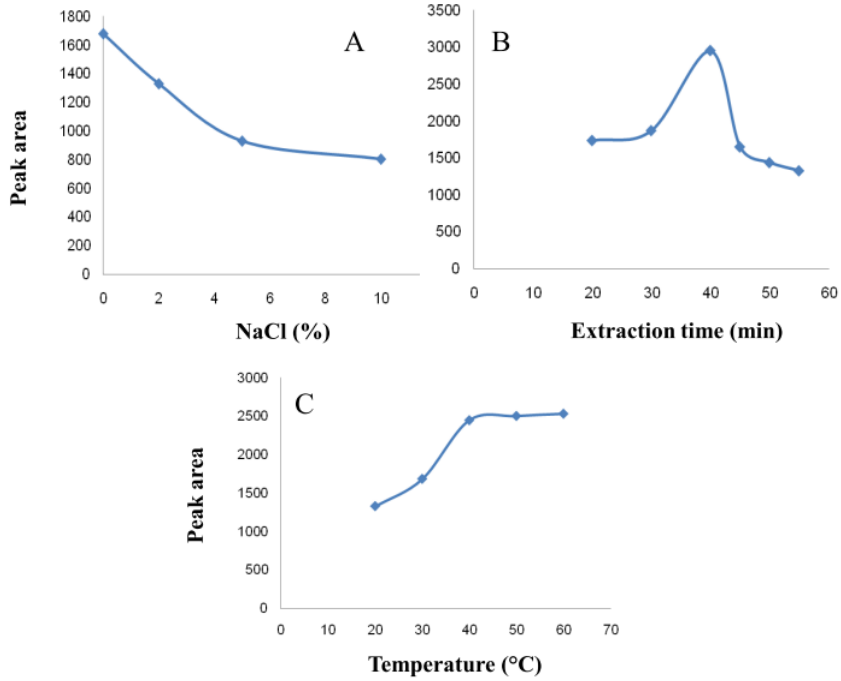


Fig. 3. Optimization of (A) salt addition effect, (B) extraction time and (C) temperature for extraction of risperidone

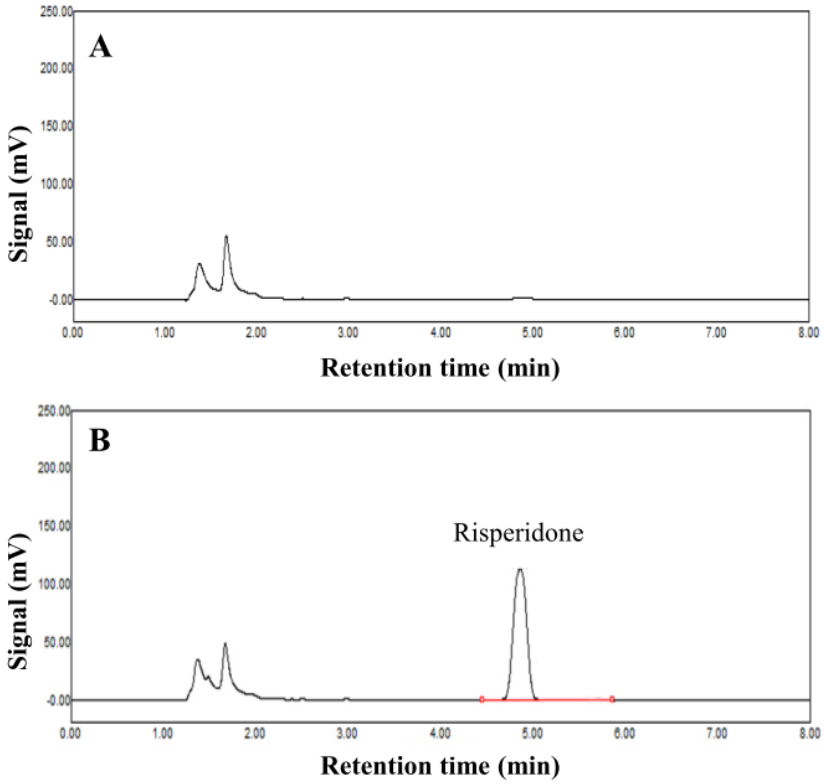


Fig. 4. Chromatograms obtained after HF-LPME extraction of urine sample ((A) non-spiked sample and (B) spiked sample at a concentration level of 1.0 mg L^{-1})

the target drug and extraction was accomplished after dilution of urine samples (1:3) and the addition of proper amount of NaOH solution to achieve pH 10. The results are summarized in Table 3. RSD% values less than 5.5% confirm the acceptable precision of proposed HF-LPME method. To evaluate the applicability of HF-LPME for human urine, a urine sample was analyzed with the proposed method. Since no risperidone was found in the sample, urine sample was spiked with the target analyte at a concentration of 1.0 mg L^{-1}. The chromatograms of HF-LPME extracts from a spiked and non-spiked samples are shown in Fig. 4.

Comparison of the proposed method with other methods

Comparison of the proposed method with different extraction methods for extraction and determination of risperidone is summarized in Table 4. It is shown that, the proposed method provided good linearity range, high repeatability, and a suitable sensitivity.

CONCLUSIONS

In the current work, a three-phase HF-LPME followed by HPLC was developed and validated for the detection and determination of risperidone in urine sample. An effective sample cleanup with a high preconcentration factor and extraction efficiency were obtained, as well as good linearity. This method is demonstrated to be a simple, sensitive and inexpensive analytical method for the determination of risperidone in real samples. Altogether, the advantages of this method will allow its facility application to the analysis of low concentration levels of risperidone in urine sample.

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