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Measurement and Preconcentration of Trace Amount of Ondansetron in Biological Samples by Liquid Phase Extraction and Solvent Bar

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Abstract

Ondansetron is an anti-nausea drug that is known to inhibit serotonin receptors (5HT3) and is used to improve or prevent nausea.

In this study, a three-phase solvent bar micro-extraction method using hollow fiber with high-performance liquid chromatography and UV detector for pre-concentration of Ondansetron in biological samples has been used. In order to achieve high extraction efficiency, different variables on extraction efficiency were optimized using Minitab software, which were receiver pH, stirring speed, extraction time, extraction temperature and salt concentration. This method successfully used on real samples such as urine and plasma.

Key Words: Ondansetron, Solvent bar micro-extraction, High Performance Liquid Chromatography, Biological Samples, Preconcentration Factor.

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Introduction

Ondansetron (Zofran), **I**) is used to prevent nausea and vomiting that may be caused by surgery, cancer chemotherapy, or radiation treatment. It blocks the actions of chemicals in the body that can trigger nausea and vomiting [1].It is also effective for treating gastroenteritis. It is a serotonin 5-HT3 receptor antagonist and does not have any effect on dopamine receptors or muscarinic acetylcholine receptors [2]. Ondansetron was patented in 1984 and approved for medical use in 1990 [3].

It is on the World Health Organization's List of Essential Medicines [4]. In 2021, it was the 79th most commonly prescribed medication in the United States, with more than 8 million prescriptions [5, 6].

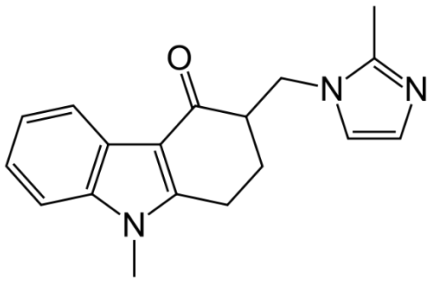


Figure 1. Structure formula of Ondansetron (**I**).

Due to the very low concentration of some pollutants and drugs in biological fluids and also due to the complexity of real samples, sample preparation is very important. Today, various techniques have replaced the classical methods, which are either solvent-free or the amount of solvent used in them is very small, and they can be considered as solvent-free methods.

Sample preparation methods can be classified into different categories based on the type of extracting phase,which some of them are as follows:

Solid phase extraction (SPE) is a general technique for extracting small amounts of pollutants in environmental samples. This method is considered one of the fast sample preparation methods and can be used for purposes such as purification, pre-concentration, desalination, derivatization, etc. The principles of this method are similar to liquid-liquid extraction and include sample distribution between two phases. This technique makes it possible to preconcentrate the analyte by absorbing it on the adsorbent. In an SPE process, contaminants and interfering agents are removed and the analyte is absorbed by the solid phase, and in the next step, the analyte is washed with a solvent from the adsorbent surface [7].

Compared to the old extraction methods, the SPE method has advantages such as high extraction speed, high concentration factor, reduced solvent consumption, solid adsorbent phase, and in

addition to high efficiency, good reproducibility and automation, it is also possible to concentrate dilute analytes. Among the disadvantages of SPE, we can mention the low recovery for complex samples and also the possibility of clogging of absorbent pores [8, 9].

The SPE method is widely used in the extraction and measurement of various compounds such as pollutants [10], drugs [11], etc. from water samples (drinking, river, etc.) [12], biological fluids (blood, plasma, serum). [13, 14], food and drinks have been used [15].

In the solid phase microextraction (SPME) method, which was invented as a solvent-free technique in 1990 by Paulgin, a very small amount of absorbent is placed on the outer surface of the fiber. In this technique, first the adsorbent is exposed to the sample, and then by establishing the balance between the sample and the adsorbent, the analyte enters the adsorbent phase and then the fiber enters the GC injection site. During the extraction and desorption stages, no solvent is used [16].

In the solvent strip microextraction method (SBME), by the hollow fiber membrane, the fiber was cut into 2 cm and the pores of the membrane and the space inside were filled with an organic solvent. Then both sides were closed and placed in the aqueous solution containing the analyte sample and a magnet stirred the solution. In this way, the analyte was extracted into the fiber and after a suitable period, the fiber was removed from the solution and its contents were injected into the analyzer [17, 18].

So far, many studies have been conducted using the SBME method to extract pharmaceutical compounds, pesticides, organic pollutants and heavy metals from different samples [19-29].

In the three-phase extraction methods that are based on the use of hollow fibers, there are three phases including sample aqueous solution, membrane organic solvent, and receiving aqueous solution. In this method, at the first, the analyte enters to organic solvent on the fiber pores and then it is extracted again into the secondary solution inside the fiber. Since the extraction is done in two steps in the current method, it has high selectivity compared to the two-phase method [30].

In this study, a three-phase solvent strip microextraction method using hollow fiber along with high-performance liquid chromatography (HPLC) and UV detector was used to preconcentrate of ondansetron in biological samples.

Experimental

Chemicals

All of the chemicals and reagents were prepared from‹Merck‹ Zibo Senlos Chem., and Sigma-Aldrich companies.

Preparation of required solutions

Initial standard solution (100 mg/l) was prepared by weighing the standard powder of **I** in methanol.Secondary standards were prepared by diluting the primary standard in deionized water. Also other standard solutions were prepared daily and freshly with appropriate dilution of standard solutions with deionized water with the desired concentration. All standard solutions were kept in refrigerator at temperature of 2-8 °C.

A plasma sample was obtained from a healthy volunteer from the Iranian Blood Transfusion Organization.

Apparatus

HPLC (Yongline), pH meter (AZ, 86502), Magnetic stirrer (HeidolPh), Micro syringe (Hamilton) and other instruments were from South Korean, Taiwan, Germany and USA.

Extraction steps

Before starting the extraction, polypropylene fibers were cut to a length of 4 cm (the volume of these fibers is 10 microliters), placed in acetone and ultrasonic bath for 10 minutes to be completely washed, then the fibers are placed at room temperature until they are completely dry for using in extraction.

In the extraction steps:

-10 ml of the sample solution was transferred to the extraction container.

- 30 microliters of the receiver phase was drawn by a 100 microliter syringe and the end of the needle was inserted into the fiber with a size of 3 mm.

-The fiber was suspended in the organic solvent (n-octanol) for 15 seconds until its pores were removed from the organic solvent

-It was saturated and then placed in distilled water for 10 seconds to wash the excess solvent from its surface.

-Receptor phase inside the syringe was injected into the fiber, and in this additional step, the receptor phase leaves the end of the fiber drop by drop.

-Two ends of the fiber were closed by a piece of aluminum foil and the fiber was suspended in the sample container and the sample solution was stirred for a certain period of time.

-At the end of the extraction time, the fiber was removed from the sample solution and its end was cut by scissors.

-Receiver phase (extract, 10 microliters) was drawn into the syringe and injected directly into the HPLC device to measure the drug.

Optimization steps

In order to investigate the effect of parameters on the amount of analyte extraction, chemometric optimization method was used.

Optimization of separation conditions

Optimization of separation conditions

In order to achieve short separation times and avoid high consumption of washing solvent as well as proper separation of analyte peaks in the chromatogram, the effect of mobile phase composition and flow rate on retention time and separation power of analytes was investigated.

Optimization of extraction conditions

In three-phase extraction based on the use of fiber, several factors influence the extraction and preconcentration rates of the analyte. Therefore, in order to optimal conditions, the effect of several factors such as the type of solvent filling the fiber pores, receiving and donor phases, the ionic strength of the donor phase, the stirring speed of the solution, the extraction temperature and also the extraction time on the extraction efficiency were investigated. In order to optimize ondansetron, a concentration of 1 mg/liter of **I** was used in an aqueous solution. To study the extraction results, the area of the analyte peaks in the chromatogram was used.

Table 1. Extraction conditions.

pH		**Ionic strength of the donor phase**	**Stirring the analyte solution**	**Extraction time**	**Temperature of the donor phase**
donor phase: 9-11	receiving phase: 2-4	0-30% v/w NaCl salt	500-1000 rpm	25-65 minutes	25-65 °C

Real sample analysis

In order to check the effectiveness of the presented method in measuring the drug in real samples, plasma and urine samples were prepared from healthy volunteers. First, 25 ml of plasma and urine were diluted to a volume of 100 ml by deionized water without adding drugs. Then by adding sufficient amount of 4HPO2K solution and sodium hydroxide, the optimum pH was obtained. This solution is used as a phaser.

Also, plasma and urine samples were prepared and extracted after increasing the standard solution and control plasma. After extraction under optimal conditions, the effect of the sample was investigated by injecting the extractive phase into HPLC and calculating the area under the peak obtained in the grading chart.

Results

Three phases are involved in three-phase microextraction, including the analyte solution phase (donor), the organic phase, and the second aqueous phase in which the extraction is performed (receiver phase). During an extraction, the desired analyte enters the organic phase under suitable conditions and then enters the receptor phase. The speed of extraction depends on the speed of mass transfer from the interfaces between donor phase/organic phase and organic phase/acceptor phase.

In SBME based on the use of fiber, the sample solution is placed in a container and the halofiber, which is closed at both ends, is placed inside the solution. Before extraction, halofiber is placed in an organic solvent to fill its pores with organic solvent. Then the excess solvent on the halofiber is washed with water. It should be said that the organic solvent must be immiscible with water. The thin layer formed by the organic phase has a thickness of about 200 micrometers, and the total organic phase used in this process is about 15 to 20 microliters. The halofiber prepared during extraction is placed inside the analyte solution. The analyte is first transferred into the organic phase located in the hollow fiber wall and then transferred to the receptor phase located inside the hollow fiber.

Optimization steps

Optimization of separation conditions

In order to achieve proper separation and symmetrical peaks, the separation conditions in the chromatography instrument were optimized. The type and proportion of solvents in the mobile phase as well as the flow rate were tested and finally the separation conditions obtained.

- Column: C_{18}, 25 cm long with an internal diameter of 4.6 mm and a particle diameter of 5 micrometers.
- Mobile phase composition: 0.02 M ammonium acetate buffer: pH 3.5 as a buffer (Solution A) and acetonitrile (solution B), which these two solutions with a ratio of 70:30.
- Washing type: isocratic
- Flow rate: 1 ml/min
- Selected wavelength: 251 nm
- Inhibition time: 7.5 minutes
- Injection loop: 10 microliters

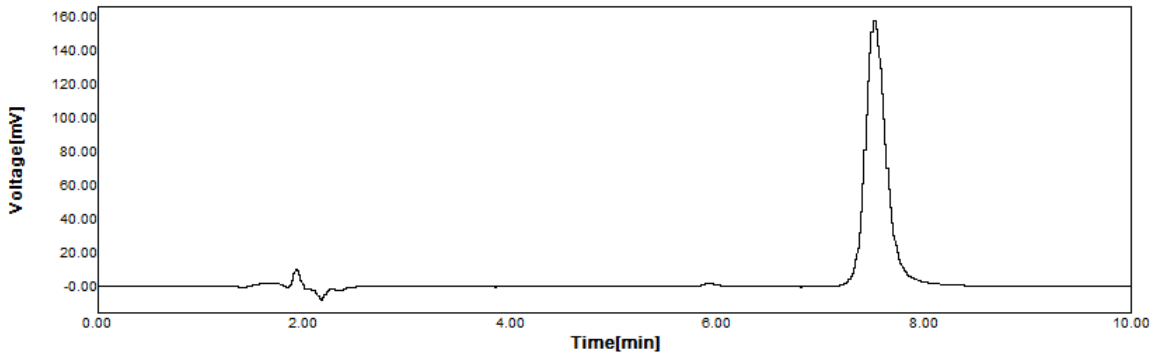


Figure 1. Direct injection chromatogram of 100mg/l ondansetron (**I**) solution.

Optimization of extraction conditions

Several factors affect the extraction with the three-phase method by porous hollow fiber, which include: type of organic solvent, pH of the donor phase, pH of the receptor phase, extraction time, stirring speed, ionic strength of the donor phase and temperature. In order to achieve the maximum extraction efficiency, high sensitivity and repeatability, it is necessary to study the effect of each of the above factors for this purpose.

Type of organic solvent

To achieve maximum extraction, sensitivity, selectivity and high accuracy, it is important to choose the right solvent. The solvent that is chosen must meet the following conditions:

- It can be easily entered into the fiber pores.
- It does not have a tendency to dissolve in the receiver and donor phases (not miscible with water).
- High boiling temperature (non-volatile).
- Appropriate viscosity (not too high so that the mass transfer of the analyte is low and not too low that it cannot remain in the fiber pores).
- Non-toxic.
- Good selectivity for combining with the desired analyte.

Experiment design

Since various factors affect the amount and efficiency of extraction and in order to achieve a high concentration factor, the effect of these factors must be investigated and optimized.

The pH of donor and recipient phases, stirring speed, ionic strength of donor phase, temperature and extraction time are among the factors that should be evaluated.

In this research, 6 mentioned factors were investigated in 3 levels and minitab software was used with the help of chemometric methods to achieve optimal conditions.

Table 2. The levels related to the examined factors in the experimental design.

Factor	Levels		
	Low (-1)	**Center (0)**	**High (+1)**
pH of donor phase	9	10	11
pH of acceptor phase	2	3	4
Stirring rate (rpm)	500	750	1000
Extraction time (min)	25	45	65
Extraction temperature (^{o}C)	25	45	65
NaCl concentration (% w/v)	0	15	30

The design including 27 experiments along with the results by minitab software for ondansetron are given in Table 3. The presented results are based on the relative sub-peak level of the species.

Table 3. Experiments designed to identify important factors affecting the results of the SBME-HF method for the isolation and measurement of ondansetron.

No	pH Ac	pH Do	Time	RPM	Salt	Temp	Area
1	2	9	25	500	0	25	820
2	2	9	25	500	15	45	1650
3	2	9	25	500	30	65	270
4	3	9	45	750	0	25	1820
5	3	9	45	750	15	45	1460
6	3	9	45	750	30	65	370
7	4	9	65	1000	0	25	290
8	4	9	65	1000	15	45	102
9	4	9	65	1000	30	65	45
10	2	10	65	750	15	25	150
11	2	10	65	750	30	45	85
12	2	10	65	750	0	65	168
13	3	10	25	1000	15	25	1700
14	3	10	25	1000	30	45	050
15	3	10	25	1000	0	65	490
16	4	10	45	500	15	25	320
17	4	10	45	500	30	45	180
18	4	10	45	500	0	65	150
19	2	11	45	1000	30	25	30
20	2	11	45	1000	0	45	80
21	2	11	45	1000	15	65	71
22	3	11	65	500	30	25	81
23	3	11	65	500	0	45	1011
24	3	11	65	500	15	65	1250

25	4	11	25	750	30	25	135
26	4	11	25	750	0	45	560
27	4	11	25	750	15	65	490

Simultaneous effect of temperature and ionic strength

With the increase of temperature and as a result of the evaporation of the organic solvent or the formation of bubbles on the fiber wall, the area under the curve in the chromatogram decreases and the extraction efficiency also decreases (Figure 2).

Also, with the increase of salt in the investigated range, the extraction efficiency increases. The reason for this is that when salt is added to the sample, the phenomenon known as out-salting occurs in such a way that water molecules form water-coating spheres around the ionic molecules of the salt, and this causes the reduction of water coating of analyte molecules and as a result the analyte molecules drift towards the organic solvent and increase the extraction efficiency.

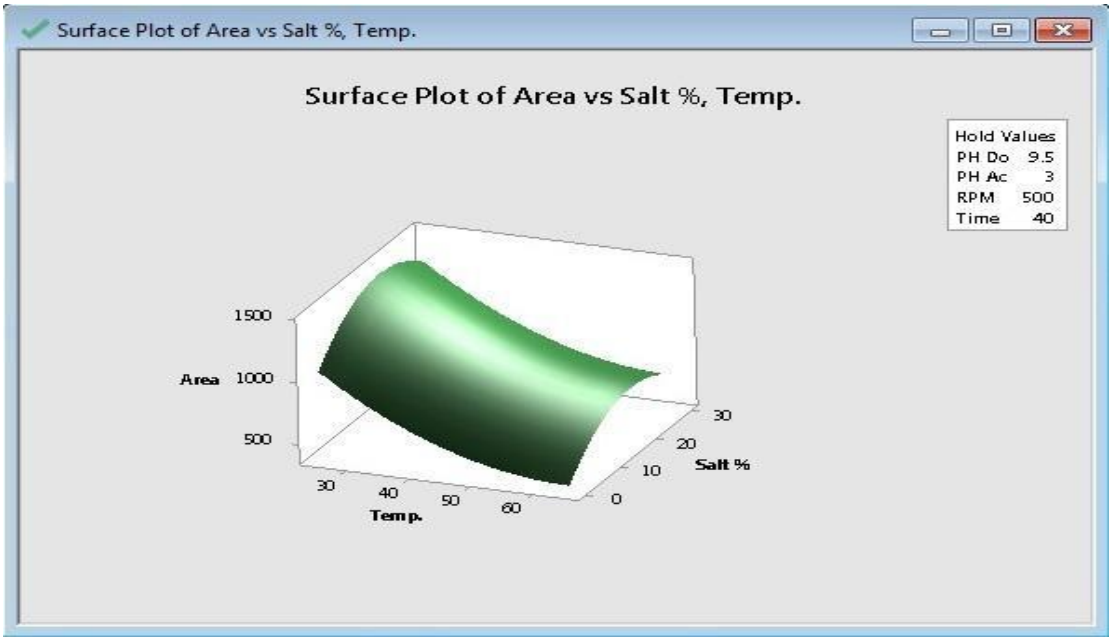


Figure 2. Simultaneous effect of temperature and ionic strength.

Simultaneous effect of ionic strength and time

As the time increases, the extraction efficiency decreases. With the increase of salt, the extraction efficiency increases and then decreases. The maximum extraction efficiency is observed in the minimum time and medium salt in the mentioned range (Figure 3).

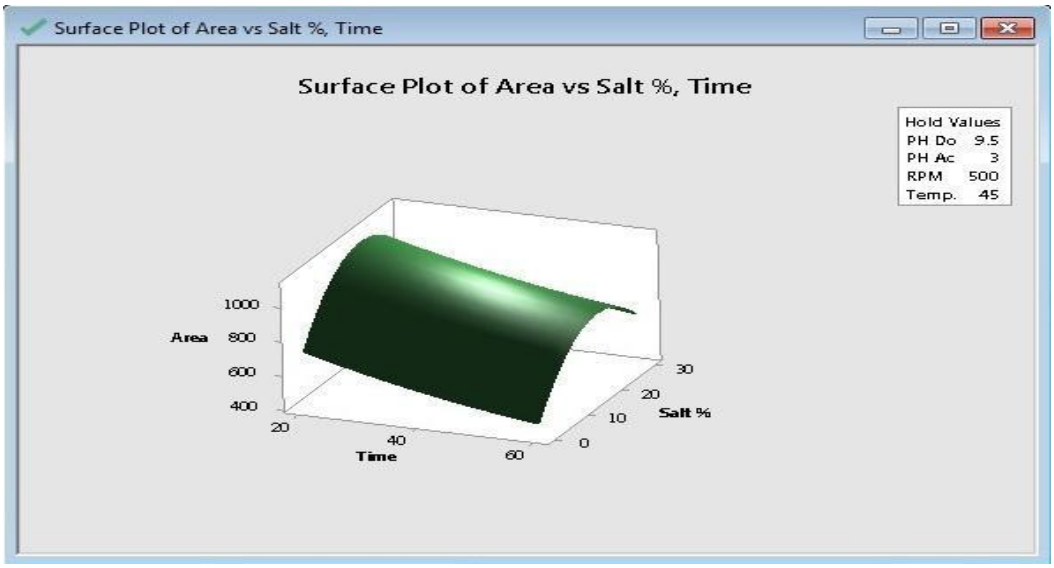


Figure 3. Simultaneous effect of ionic strength and time.

Simultaneous effect of temperature and time

As seen in Figure 3, the extraction efficiency decreases with increasing time. Also, with an increase in temperature, we will have a decrease in the area under the curve. As a result, the maximum extraction efficiency is obtained at the lowest level of time and temperature in the investigated range.

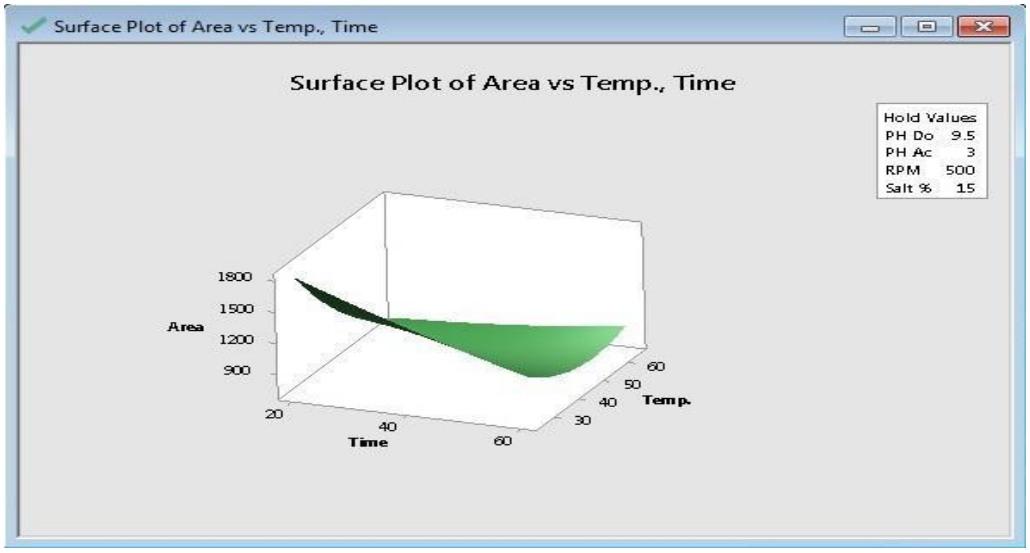


Figure 3. Simultaneous effect of temperature and time.

Simultaneous effect of temperature and stirring speed

As can be seen in Figure 4, the area under the curve increases with the increase in stirring speed. Three-phase microextraction using fiber is based on establishing balance between three phases. The overall speed of extraction is limited in two stages of analyte transfer at the interface of donor phase - organic phase and organic phase - acceptor phase. Stirring the solution is usually used to improve

the kinetics and reduce the extraction time, because by stirring the solution, the equilibrium between these two phases is quickly established. In this research, a magnetic stirrer was used to stir the solution. In this method, when the rotation speed is too high, due to the creation of a vortex state in the sample solution, it causes bubbles on the fiber, which leads to a decrease in the transfer of analyte into the fiber and ultimately a decrease in the repeatability and accuracy of the work. By increasing the temperature in the investigated range, we will decrease the extraction efficiency. The maximum extraction efficiency is at the maximum RPM and close to the minimum temperature.

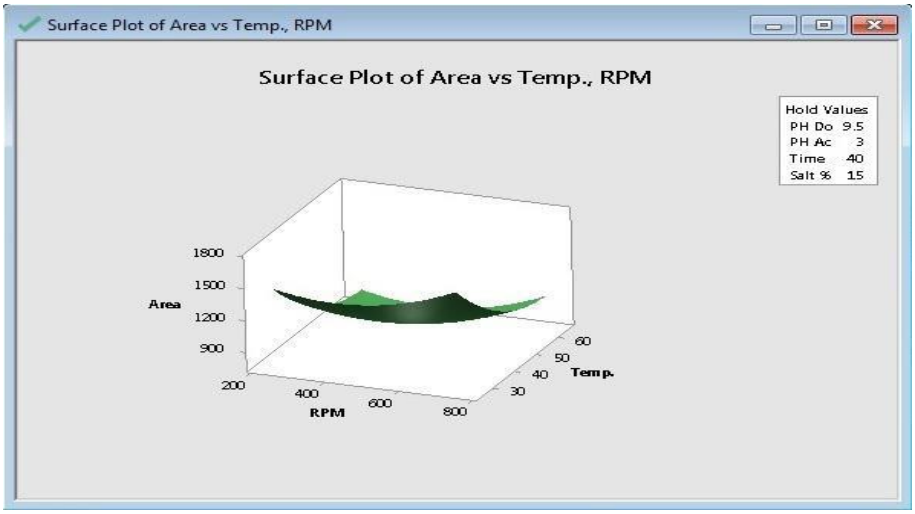


Figure 4. Simultaneous effect of temperature and stirring speed.

Simultaneous effect of stirring speed and time

As can be seen in Figure 5, the extraction efficiency increases with the stirring speed. The maximum extraction efficiency is investigated at the lowest level of time and maximum RPM.

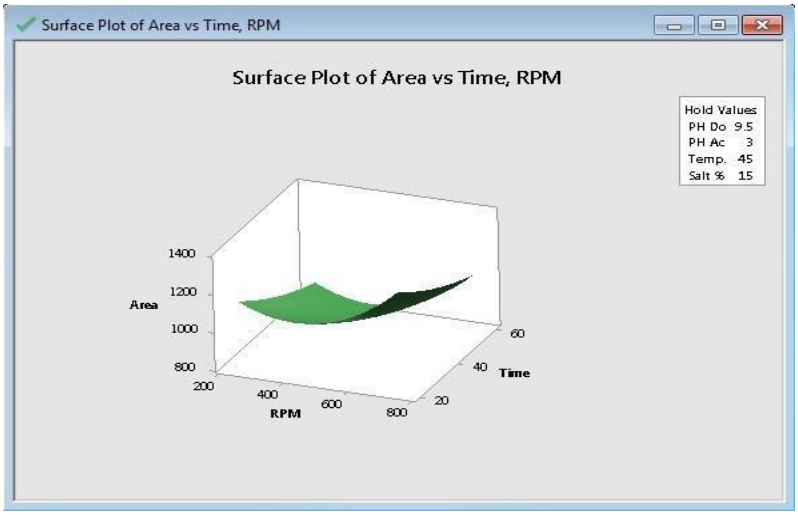


Figure 5. Simultaneous effect of stirring speed and time.

The simultaneous effect of ionic strength and pH of the receiver phase

As can be seen in Figure 6, at acidic pH, the target analyte is ionized at the internal contact surface of the organic phase and the receiver phase and enters and shows good extraction in this area. With the increase of salt, the extraction efficiency first increases and then decreases. The maximum extraction efficiency is near the average limit of ionic strength and pH of the receiver phase in the investigated range.

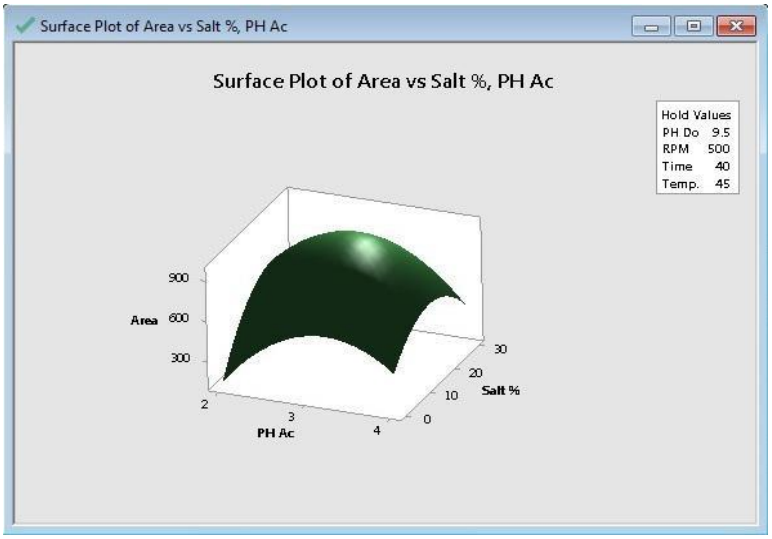


Figure 6. The simultaneous effect of ionic strength and pH of the receiver phase.

Simultaneous effect of temperature and pH of the receiver phase

As seen in Figure 7, with the increase in the pH of the receiver phase, the area under the curve will increase to an average level and then decrease. As the temperature increases in the investigated range, the area under the curve decreases. The maximum extraction efficiency is near the minimum temperature and average pH of the receiver phase in the investigated range.

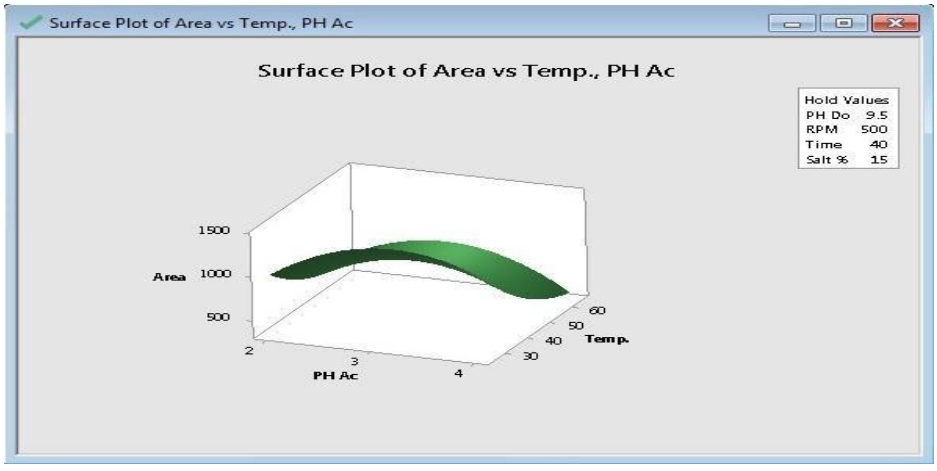


Figure 7. Simultaneous effect of temperature and pH of the receiver phase.

The simultaneous effect of pH of the receiver phase and time

As seen in Figure 8, increasing the pH of the receiver phase, increases extraction and then decreases it. Likewise, the extraction efficiency decreases with increasing time. The maximum extraction efficiency is in the minimum time and the average pH of the receiving phase is within the investigated range.

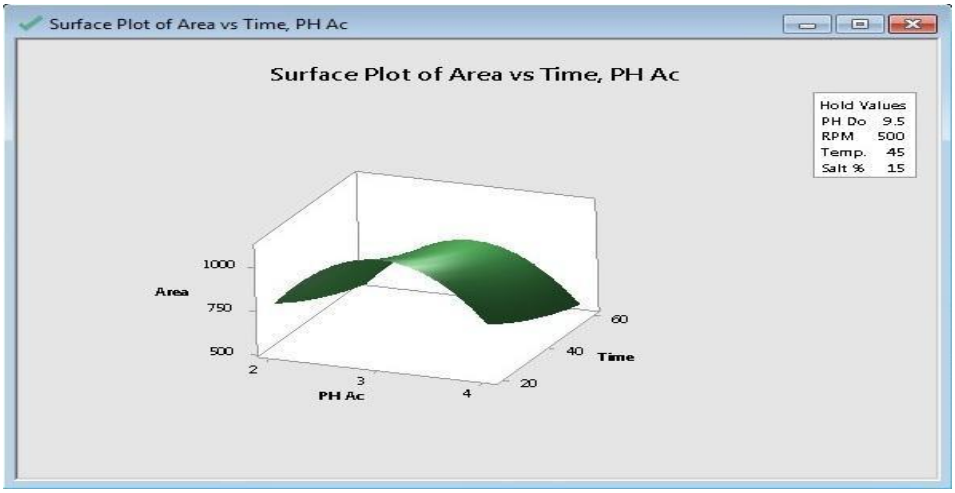


Figure 8. The simultaneous effect of pH of the receiver phase and time.

Simultaneous effect of stirring speed and pH of the receiving phase

As seen in Figure 9, with increasing the pH of the receiver phase, extraction increases and then decreases. The maximum extraction efficiency is near the maximum stirring speed and the average pH of the receiver phase in the investigated range.

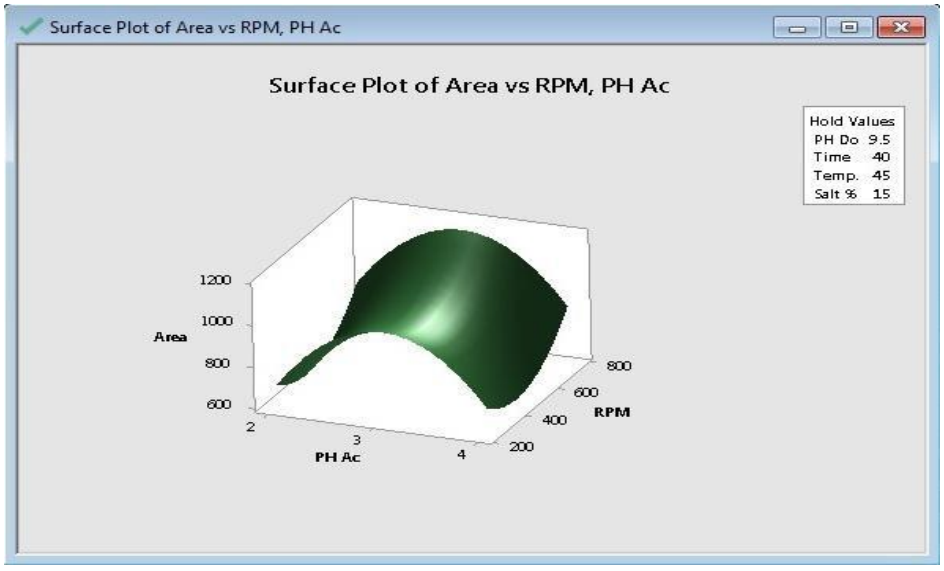


Figure 9. The simultaneous effect of pH of the receiver phase and time.

Simultaneous effect of donor phase pH and ionic strength

As can be seen in Figure 10, with the increase in the pH of the donor phase, the extraction efficiency first increases and then decreases. The pH of the donor phase must be adjusted in such a way that it can effectively deionize the desired analyte and then increase the extraction of the analyte into the organic solvent by reducing the solubility in water. With the increase of salt, the extraction efficiency increases. The maximum extraction efficiency is near the average pH of the donor phase and the maximum salt content is in the investigated range.

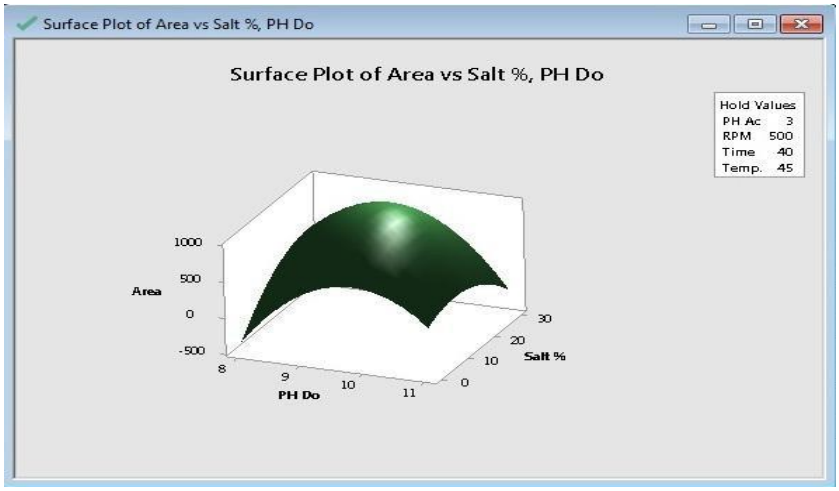


Figure 10. Simultaneous effect of donor phase pH and ionic strength.

Simultaneous effect of temperature and pH of the donor phase

As can be seen in Figure 11, the area under the curve will increase with the increase in the pH of the donor phase and the area under the curve will decrease with the increase in temperature. The maximum extraction efficiency is observed near the average pH of the donor phase and the minimum temperature in the investigated range.

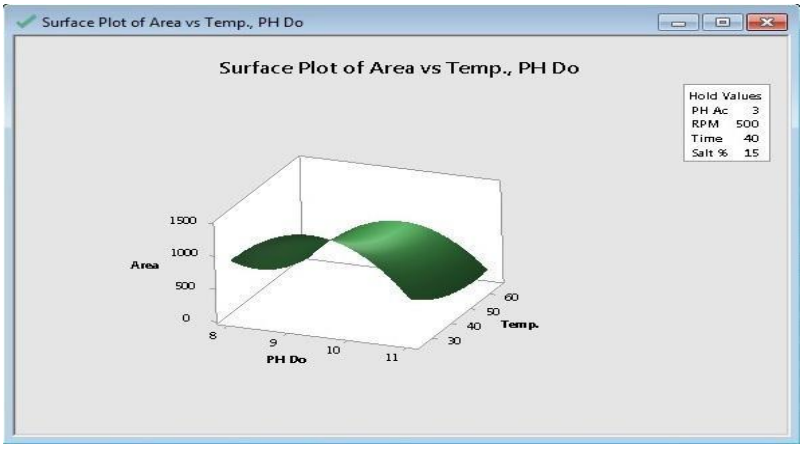


Figure 11. Simultaneous effect of temperature and pH of the donor phase.

Simultaneous effect of donor phase pH and time

As can be seen in Figure 12, by increasing the pH of the donor phase, extraction efficiency will increase to the moderate level. Also, with the increase of time for the mentioned reasons, the efficiency of extraction will decrease. The maximum extraction efficiency is observed near the average pH of the donor phase and the minimum time is observed in the investigated range.

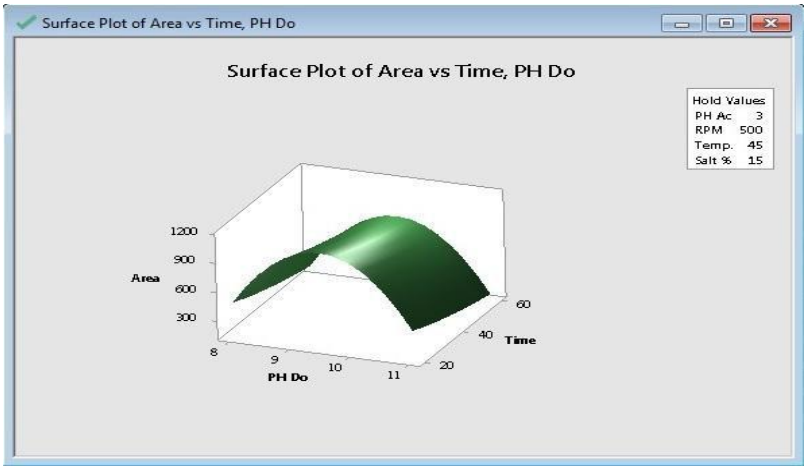


Figure 12. Simultaneous effect of donor phase pH and time.

The effect of stirring speed and pH of the donor phase

As can be seen in Figure 13, increasing the pH of the donor phase, at the first increase and then decrease was seen in the area under the curve in chromatogram. Also, by increasing the stirring speed for the reasons mentioned above, the extraction efficiency will increase. The maximum extraction efficiency is observed at the medium pH of the donor phase and close to the maximum RPM in the investigated range.

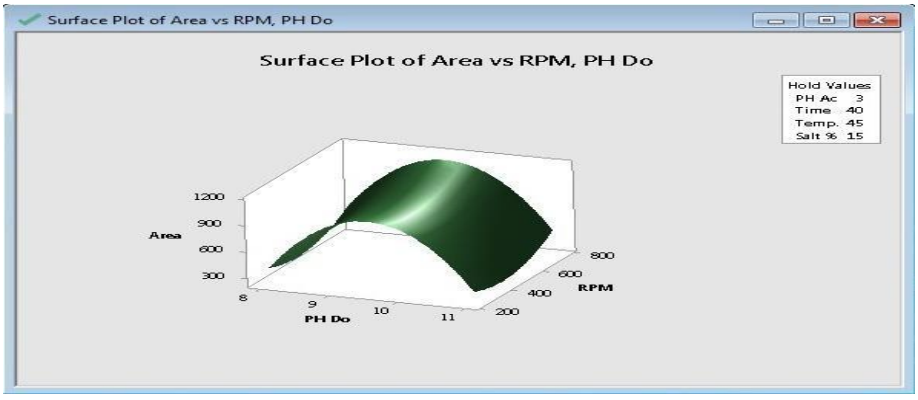


Figure 13. The effect of stirring speed and pH of the donor phase.

Simultaneous effect of ionic strength and stirring speed

As can be seen in Figure 14, by increasing the salt to an average level, an increase in the area under the curve in the chromatogram was seen. Also, with the increase in RPM, an increase in the area under the curve was observed due to the mentioned reasons.

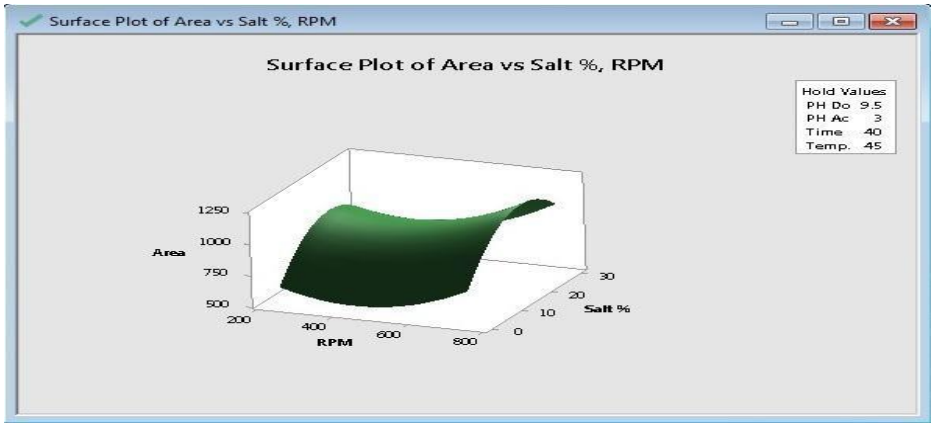


Figure 14. Simultaneous effect of ionic strength and stirring speed.

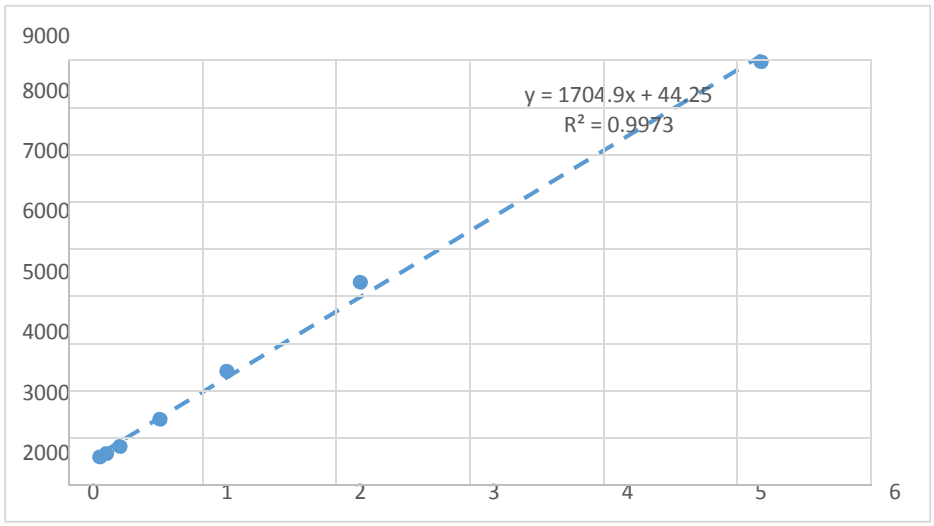
The maximum extraction efficiency is observed near the average ionic strength and the maximum rotation speed in the investigated range. According to the results and peaks obtained by minitab software, the best extraction conditions were obtained.

Table 4. Optimum conditions for ondansetron extraction.

Temp. (°C)	Extrac. Time (min.)	Ionic Strength NaCl (w/v%)	R.P.M	pH Receiver Phase	pH Donor Phase	Organic Solvent
25	20	19.09	750	3.15	9.27	n-octanol

Preparation of calibration curve

Standard solutions with concentrations of 0.05, 0.1, 0.5, 1, 2, 5, and 10 mg/liter of the drug were prepared for drawing the calibration curve. Then extraction was done under optimal conditions from the prepared standard solutions. Finally, the obtained sub-peak level was drawn according to the initial concentration of the drug in the donor phase. As can be seen, the calibration curve after extraction in water is linear with a correlation coefficient of 0.9977.



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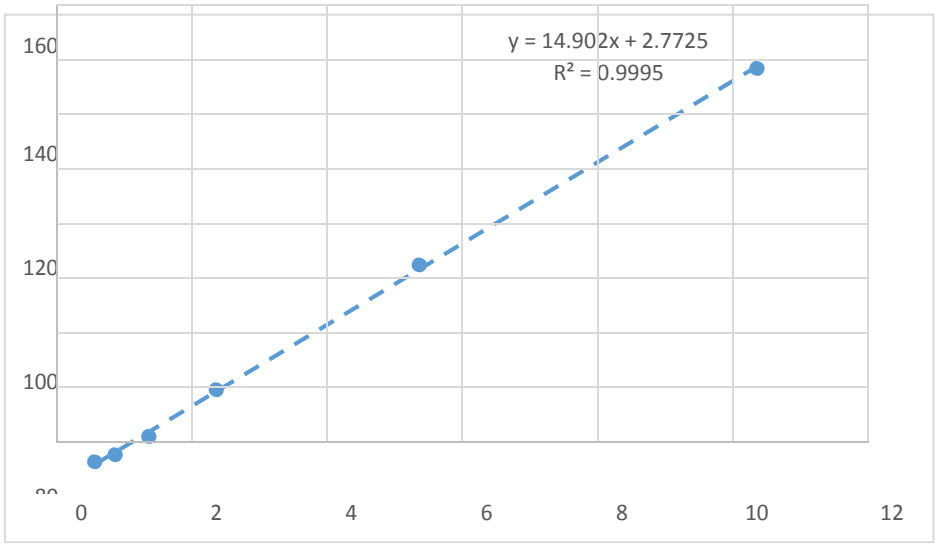


b

Chart 1. Calibration curve before (a) and after (b) extraction in aqueous solution.

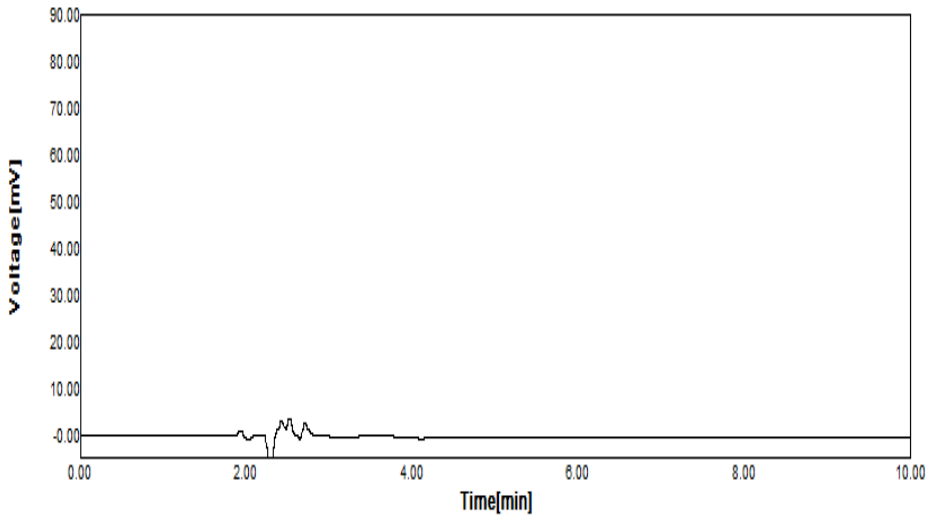
At this stage, to validate the extraction method, its analytical parameters, including repeatability (RSD), preconversion factor (PF), recovery percentage (%R) and limit of detection (LOD) were investigated.

Table 5. Optimal analytical parameters of the extraction method.

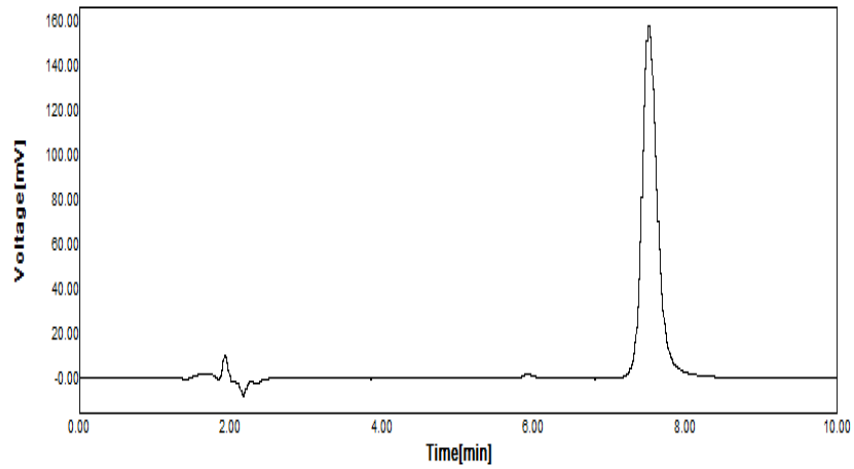
LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Linearity (ng mL^{-1})	R^2	PF	RSD%	
					Intra	Inter
15	50	50-5000	0.996	114	4.9	5.3

Real sample analysis (plasma and urine)

To check the efficiency of the mentioned extraction method, a biological sample of plasma and urine of a healthy person was used. A specific volume of plasma and urine was taken and diluted 1:4 with deionized water with HPLC purity, then its proteins were precipitated with 35% perchloric acid and put it on a vortex for 1 minute. Then centrifuges for 6 minutes at 3000 rpm. The supernatant solution was removed and its pH was adjusted to 11 by NaOH adding and its salt by adding of NaCl (30%). This solution was used as a donor phase. Also, plasma and urine samples were prepared by increasing the standard with a concentration of 1 mg/l, and preparation and extraction were also done similar to the control (plasma and urine).

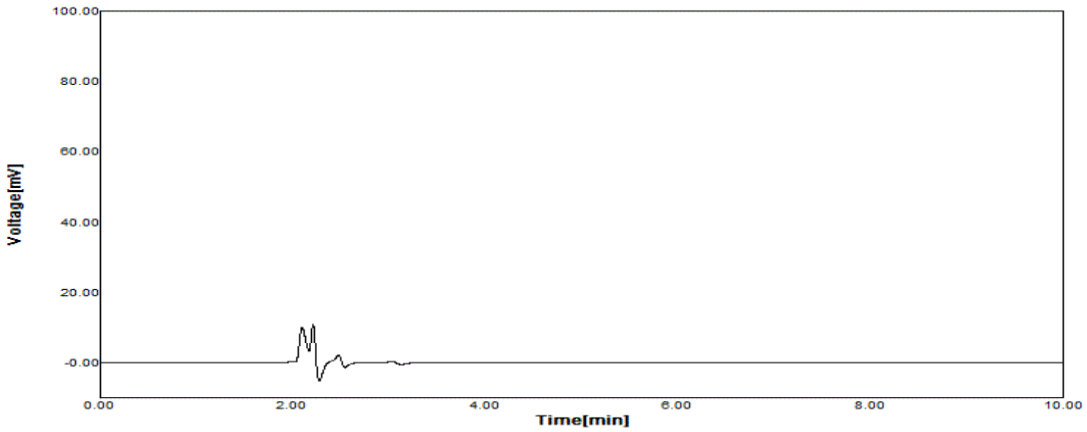


a

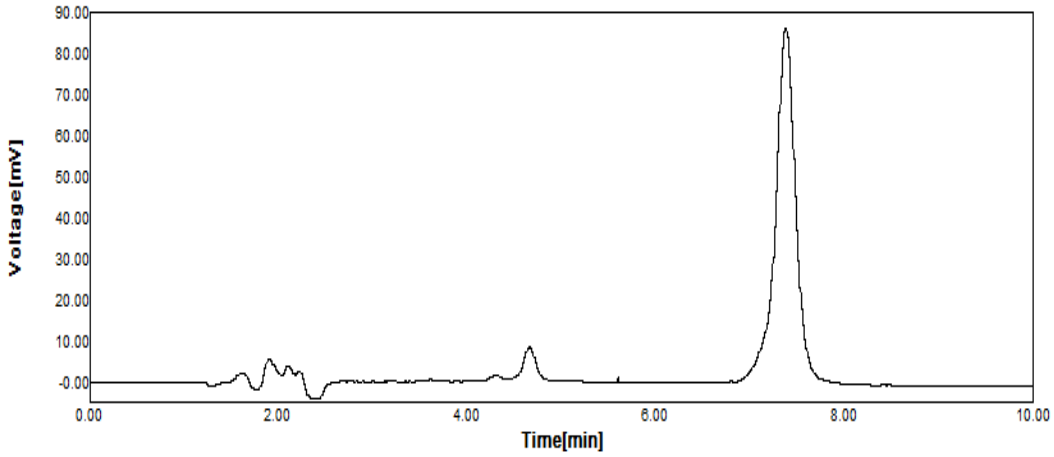


b

Figure 15. Chromatogram of control plasma sample (a) and plasma sample containing drug (b).



a



b

Figure 16. Chromatogram of control urine sample (a) and urine sample containing drug (b).

Table 6. The results of ondansetron in urine and plasma.

Sample	C_{real} (ngmL^{-1})	C_{added} (µg mL^{-1})	C_{found}(µg mL^{-1})	RSD% (n = 5)	RR %
Plasma	nd[1]	1	0.88	6.5	88
Urine	nd	1	0.91	5.7	91

Discussion

The two LLE and SPE methods, despite good repeatability, desirable concentration and high sample capacity, also have disadvantages, which could be mentioned below:

1. The methods are laborious, expensive and tiring.

2. Both techniques need to remove the solvent, LLE (with solvent evaporation) and SPE (with solvent washing), which results in the loss of the analyte sample.

3. The LLE method requires a large volume of organic solvent (with high purity), which in addition to the risk of inhalation and skin contact with the solvent, creates toxicity in laboratory wastes and damages the environment.

4. The LLE method for sample preparation which is a multi-step method, may lose analyte during the process.

5. In LLE, the low recovery percentage of analyte, the extraction of impurities at the same time and also the possibility of emulsion formation were seen.

6. The new generation of SPE techniques, which consume less solvent, are very laborious and time-consuming methods and have very expensive automation.

These disadvantages have led to the use of SBME method in this research to extract ondansetron, which has the following advantages:

1.In SBME, organic solvent is consumed much less than other mentioned methods that is one of the most important advantages of this method.

2. Due to the very small volume of the extraction phase (in the range of microliters), there is no need for a pre-concentration step before the final analysis and the extraction phase can be directly injected into the HPLC device.

3. Consumable fiber acts as a filter due to having small pores, which prevents the entry of large molecules and particles in biological samples into the organic phase and as a result, the method has a high cleaning power. Therefore, this technique can be easily used to extract different types of complex tissues.

4. Due to the simplicity and low price of extraction tools, fresh fibers are used for each fiber extraction.

5. In addition to high accuracy and sensitivity, this method has advantages such as low consumption of sample and organic solvent and adequate extraction time.

6. The extraction phase is not in direct contact with the sample phase, as a result, the sample phase can be stirred at any intensity without worrying about losing the extraction phase.

7. The method is very economical.

8. The method has very high power and ability to filter and extract from the sample. SBME is an equilibrium extraction technique which concentration of the analyte in the phase receiver increases to a certain level and then the system is generally balanced and the concentration of the analyte remains constant over time in the receiver phase.

9. Extraction can be done in a wide range of pH, while other methods lack this ability.

10. When the analyte is extracted from a relatively large volume to a small volume of the receiving phase, large pre-concentrations are obtained and the whole extraction and pre-concentration steps take place in one step and no needs to removed organic solvent.

11.This method is more common than LC/MS and radioimmunoassay methods and can be performed in normal laboratories.

Conclusion

In this study, a three-phase solvent bar micro-extraction method using hollow fiber with high-performance liquid chromatography and UV detector for pre-concentration of Ondansetron in biological samples has been used. In order to achieve high extraction efficiency, different variables on extraction efficiency were optimized using Minitab software which were: the pH of the donor 9.27, the pH of the receiver phase 3.15, the speed of stirring, 750 rpm, the extraction time 20 min, the extraction temperature 25° C, and 19.09% salt percent. The method then successfully used on real samples such as urine and plasma.

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