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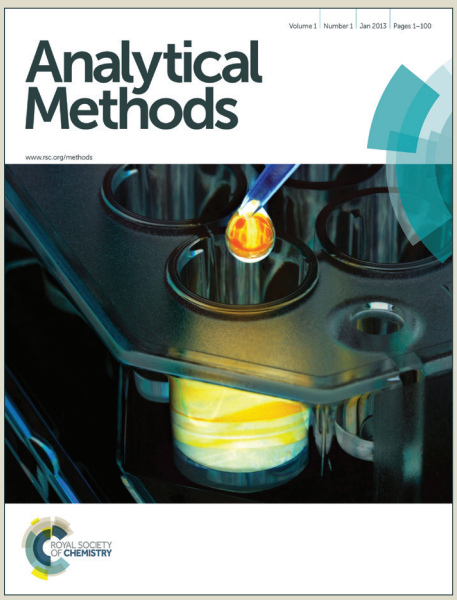

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ARTICLE

Evaluation of three-phase hollow fiber microextraction based on two immiscible solvents coupled to GC and HPLC for determination of statin drugs in biological fluids

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In this study, for the first time extraction of three statin drugs from biological fluids using hollow fiber liquid phase microextraction) based on two immiscible organic solvents prior to high performance liquid chromatography with diode array detection and gas chromatography with flame ionization detector was investigated experimentally and theoretically. Three major statin drugs; atorvastatin, lovastatin, and simvastatin, were first extracted from sample solution, into a thin layer of organic solvent (5% trioctylphosphine oxide in *n*-dodecane) sustained in the pores of a porous hollow fiber, and further into a µL volume of organic acceptor (alkaline methanol) located inside the lumen of the hollow fiber. A systematic investigation of the proposed method was applied to find optimal extraction conditions and evaluate the interaction effects between the factors by designing experiments according to the methodology of central composite design. Under the optimized conditions, this technique provided preconcentration factor in the range of 466 to 863-fold and 550 to 880 for HPLC and GC analysis, respectively. Good precisions values (with RSDs ≤ 12.1%) were obtained. Detection limits were in the range of 0.2 – 3 µg L^{-1} and 0.2 – 5 µg L^{-1} for the water and biological samples by HPLC and GC, respectively. Also, quantification limits were in the range of 0.5 – 5 µg L^{-1} and 1 – 10 µg L^{-1} for HPLC and GC in water and biological samples, respectively. Extraction recoveries of the drugs in water, urine, and plasma samples were obtained in the range of 64.7 – 110.0 % and 61.2 – 104.4 % by HPLC and GC, respectively. Comparing with the traditional methods, the proposed method exhibits high clean up with suitable sensitivity and high preconcentration factors as well as good precision.

1. Introduction

Lipid regulating agents can be divided into two main groups namely "the fibrate" and "the statin" class. In contrast to the extensive information related to the fibrate class in the environment, only a few papers have been published on the presence of pharmaceuticals belonging to the statin class [1]. Statins include natural (lovastatin), semi-synthetic (simvastatin and pravastatin), and synthetic compounds (fland synth, atorvastatin, cerivastatin, rosuvastatin, and pitavastatin). Moreover, the ''statin'' class of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are highly effective in reducing total cholesterol and low-density lipoprotein (LDL) cholesterol levels in the human body [2]. Atorvastatin, lovastatin, and simvastatin are the most globally available commercial statin formulations used in the clinical treatment of hypercholesterolemia. The therapeutic range of statins is typically 10 – 80 mg day^{-1}. All statins are absorbed rapidly following administration, reaching the peak plasma concentration within 4 hours. The maximum plasma concentration has been reported to be 27 – 66 µg L^{-1} for atorvastatin and 10 – 34 µg L^{-1} for simvastatin [3, 4]. High doses could be used with caution in the elderly, in patients with

renal or hepatic insufficiency, hypothyroidism, or diabetes [5]. Therefore, it would be highly convenient and helpful to monitor the statin levels in biological fluids in order to establish and control appropriate dosage scheme, which would minimize the side effects and keep the cholesterol lowering effect.

Different techniques, including high performance liquid chromatography (HPLC) together with various types of detection methods, including UV (ultraviolet) detection [6], FD (fluorescence detection) [7], and MS (mass spectrometry) [8] have been applied for determination of different statin drugs. Gas chromatography (GC) is somewhat suppressed because of the need for a special conditions for analysis of drug molecules, which is often not volatile in pharmaceutical formulations.

In general, sample preparation and concentration of the target analytes are often needed before analysis. Only a few methods have been designed for separation of the statin mixtures [1, 9]. For instance, liquid-liquid extraction (LLE) has been used for separation of atorvastatin [10], while there are some reports on the use of solvent extraction for the analysis of lovastatin in the fermentation broth [11], and solid phase extraction (SPE) for simvastatin and simvastatin acid in human plasma [12] to purify samples before analysis.

LLE has been the primary sample preparation method that is still very popular and has been widely applied to the sampling and analysis of various samples [13-15]. Other popular sample preparation procedures are solid-phase extraction (SPE) and solid-phase microextraction (SPME), which were commercially introduced and satisfy most requirements of a good sample preparation technique. However, there are some limitations, such as the quality of the sorbents or fibers depending on the commercial supplier and sample carry-over [16, 17]. In recent years, the so-called microextraction techniques have become popular as sample preparation techniques. Among microextraction methods, Hollow fiber based liquid-phase microextraction (HF-LPME) is an efficient alternative to the classical techniques for sample preparation and preconcentration to minimize organic solvent usage and to overcome the problems of conventional preconcentration methods such as requiring relatively high sample volume, multi-step extraction process, and consuming some toxic solvents as the eluent. To date, HF-LPME approaches that have been developed include two-phase and three-phase HF-LPME. In the two-phase HF-LPME, the analytes are extracted by passive diffusion from the sample into a hydrophobic organic solvent, supported by the fiber. In three-phase HF-LPME, the analytes are first extracted from the aqueous solution into a thin layer of an organic solvent (several microliters) immobilized within the pores of the fiber, then further extracted into an aqueous acceptor phase inside the lumen of the fiber [18]. Another mode of the three-phase HF-LPME introduced by Ghambarian et al. is based on using two immiscible organic solvents [19-21]. Here, an organic solvent (*n*-dodecane) is immobilized in the pores of the hollow fiber, providing a supported liquid membrane (SLM), and the other organic solvent (acetonitrile or methanol), which is immiscible with *n* -dodecane is filled in its lumen. In another similar work, Basheer et al. reported a three-phase mode of HF-LPME that involved using an immiscible ionic liquid and organic solvent as SLM and acceptor phase, respectively [20]. The main advantages of HF-LPME is a high degree of clean-up, especially in complex matrices such as biological fluids, high preconcentration factors, as well as a good selectivity by selection of proper organic solvents.

The aim of the present study was to affirm the high extraction efficiency of HF-LPME based on two immiscible organic solvents compared with the aqueous acceptor phase for extraction of three common statin drugs (atorvastatin, simvastatin, and lovastatin). Because of using organic solvent as acceptor phase, the quantitative analyses could be performed by GC-FID in addition to HPLC for the first time. In the present work, a central composite design was used to

optimize the values of the variables significantly affecting the HF-LPME conditions in order to obtain the best response. In this way, the interactions of parameters and the curvature among experimental variables are studied and, therefore, a real optimum is achieved. This chemometrics approach allows the simultaneous variation in all the factors affecting the experiment and evaluation of interactions among them. After this, the method performance is evaluated in terms of linearity, precision, accuracy, and limits of detection. Finally, the method is applied to the analysis of real biological samples that obtained from persons who used some of these drugs.

2. Experimental procedures

2.1. Materials and supplies

Atorvastatin, simvastatin, and lovastatin were obtained from the Tehran Chemie Pharmaceutical Co. (Tehran, Iran). HPLC-grade acetonitrile and methanol were purchased from Samchun (Pyeongtaek, South Korea). Trioctylphosphine oxide (TOPO) was purchased from the Sigma-Aldrich (St. Louis, MO, USA). The ultrapure water was used for preparation of stock standard and sample solutions. The pH of the solution was adjusted with 1 mol L^{-1} nitric acid or sodium hydroxide solution. All the other chemicals used were of reagent grade or the highest purity available. The Accurel Q3/2 polypropylene HF membrane (600 mm id, 200 mm wall thickness, and 0.2 mm pore size) was supplied by Membrana (Wuppertal, Germany). The plastic and glassware used for the experiments were previously soaked in nitric acid (1 mol L^{-1}) then ultrapure water and acetone rinsed carefully with the ultrapure water.

2.2. Biological samples and standard solutions

Urine and plasma samples containing the drugs were obtained from two patients, all samples were collected in accordance with the ethical guidelines and permission of institutional review board (IRB), and stored at 4 ºC and -20 ºC, respectively. The drug-free plasma and urine samples were obtained from the Hakim Medical Clinic (Tehran, Iran) as a match matrix for plotting the calibration curves. The pH values of the real samples were adjusted at 2.0 by dropwise addition of 1 mol L^{-1} HNO_3 solution. The separate stock standard solutions containing 400 mg L^{-1} of simvastatin, atorvastatin, and lovastatin were prepared by dissolving appropriate amounts of the drugs in methanol. All stock solutions were stored at 2 – 8 ºC. The stock solutions were further diluted with the methanol–water (1:1, v/v; pH adjusted to 2.0 with nitric acid) to give the standard mixture having a final concentration in the range of 0.2 – 500 µg L^{-1}.

2.3. Instrumentation

An Agilent HPLC instrument (Wilmington, USA) equipped with Agilent G1315D diode array detector with a 1200 series quaternary pump and an AgilentEclipse-XDB-C18 analytical column (250 mm × 4.6 mm, 5 µm) was applied to separate and detect the analytes. The aqueous mobile phase was initially composed of 30% methanol (component *A*) and 30 mmol L^{-1} phosphate buffer with pH 4.5 (component *B*). During the run of solvent gradient program, from the first 2 to 15 min, the concentration of component *A* was linearly increased to 100%, where it was held for 10 min. The mobile phase flow rate was 1 mL min^{-1}, and the injection volume was 20 µL. Diode array detection (DAD) monitoring wavelength was 247 nm for all of the analytes. Gas chromatographic analyses were performed on an Agilent 7890 gas chromatograph (Centerville Road, Wilmington, NC, USA) equipped with a flame ionization detector (FID). Separation of the analytes was performed in HP-5 fused silica capillary column (30 m×0.32 mm id, 0.25 mm film thickness) from Supelco (Bellefonte, PA, USA). The temperature of the detector was 300º C and it was fed with 35 mL min^{-1} of hydrogen, 350 mL min^{-1} of air, and 20 mL min^{-1} of nitrogen as auxiliary gas. Also, 2 µL aliquot of the extract was autoinjected by Agilent series 7683 automatic liquid sampler (Centerville Road, Wilmington, USA). The injection port was

operated at splitless mode and its temperature was adjusted at 300º C. Helium (with 99.999% purity) was used as carrier gas with a constant column flow of 2 mL min^{-1}. The oven temperature program was 50º C for 2 min, increased to 200º C at 20º C min^{-1}, held for 0 min, increased to 280º C at 10º C min^{-1}. The laboratory apparatus used included a digital IKA vortex (MS3 basic) (Deutschland, Germany), a Wise Stir®MSH-D plate magnetic stirrer (Seoul, Korea) for stirring the sample solutions and a parsia Ind group centrifuge (Tehran, Iran) to separate the supernatant layer from the precipitated plasma proteins. The pH of solutions was measured by a Metrohm 781 ion analyzer (Herisau Switzerland) supplied with a combined glass-calomel electrode.

2.4. Calculations

The preconcentration factors (*PF*) and extraction recoveries (*ER*) of drugs were calculated based on the following equations:

$$PF = \frac{C_{a,final}}{C_{d,initial}} \quad (1)$$

$$\%ER = (\frac{V_a}{V_d})PF \times 100 \quad (2)$$

V_a and V_d are volumes of the acceptor and donor phases, respectively, $C_{a,final}$ is the final concentration of the analyte in the acceptor phase, and $C_{d,initial}$ is the initial analyte concentration in the donor phase. Relative recovery (*RR*) was acquired from the following equation:

$$\%RR = \frac{C_{found} - C_{real}}{C_{added}} \times 100 \quad (3)$$

where C_{found}, C_{real}, and C_{added} are the analyte concentration after addition of a known amount of standard into the sample, the analyte concentration in the sample, and the concentration of a known amount of the standard that was spiked into the sample, respectively.

2.5. Extraction procedure

A certain volume of the aqueous sample (18.0 mL) was placed in a 20-mL sample vial. The sample vial position was fixed above the magnetic stirrer. Polypropylene hollow fibers were cut into 10-cm pieces. The fibers were immersed in the membrane phase (5% TOPO in *n*-dodecane) for 10 s to impregnate the fiber pores. Outside of the fiber was then rinsed with water to remove any excess organic solvent. The lumen of the prepared fiber piece was filled with 25 µL of the acceptor phase (0.1 mol L^{-1} NaOH in methanol) using an HPLC microsyringe. Both open ends of the fiber were then attached to the 25-µL HPLC microsyringe and a conventional medical syringe needle. During extraction in a 20-mL sample vial, the U-shape membrane portion containing the acceptor phase was immersed in 18 mL of the sample solution (pH 2.0). The sample solution was stirred at 1000 rpm for 45 min. After extraction, U-shape set-up was taken out of the solution and one end of the fiber was detached from the medical syringe needle. The acceptor phase was then withdrawn into the HPLC microsyringe and 20 µL and 2 µL of the extracted solution was injected into the HPLC and GC system, respectively. It should be noted that the final collected acceptor phase was in the range of 22-24 µL.

Experimental design approach

The statistical computer package "Statgraphics Plus 5.1" (Manugistics Inc, MA, USA) was used. The software statistically analyzes the experimental data (e.g., ANOVA, determination of the estimated effects and interaction, regression equation, which has been fitted to the data) and plots the Pareto chart and the estimated response surface.

2.6. A response surface design for optimization of the parameters

A central composite design (CCD) was built using all the variables that can affect the extraction. The total number of experiments (N) with *f* factors is:

$$N = 2^f + 2f + N \quad (4)$$

The first term is related to the full factorial design, the second to the star points, and the third to the center point. The length of the arms of

the star (α) played a major role in the appearance of the CCD. Based on α value, there are two different designs for CCD:

Face centered central composite design (FCCCD) with |α| = 1 and circumscribed central composite design (CCCD) with |α|>1 were used to investigate the variables at three and five levels, respectively. All other experiments were performed randomly and without replication. Finally, the response surface plots were presented for visualization and rapid selection of the optimal conditions. Response surface plots are presented in three dimensional spaces and clearly show influence of the factors on the extraction efficiency in the investigated region and also slightly outside of the investigated region [23].

3. Results and discussion

3.1. Optimization of the HF-LPME procedure

Different parameters such as HF length, type of the acceptor phase and SLM, pH of the donor and acceptor phases, stirring rate, extraction time, and the effect of salt addition can affect the statin extraction efficiency. Investigation of pH of the donor and acceptor phases, type of the organic membrane, and type of the organic acceptor solvent in screening design experiments is time consuming and increases the number of runs. Study the effect of this parameter separately can reduce the number of runs. Therefore, these parameters were evaluated using single dimensional search, so that every related single factor was varied whilst all other factors were kept fixed at a specific set of conditions. Other effective parameters such as HF length, extraction time, salt addition, and stirring rate were assessed and optimized with the aid of the response surface methodology. A second-order model relating the peak area with significant independent variables was conducted by CCD. Then, a model for the response was built and the optimal conditions were predicted.

3.1.1. Acceptor phase and SLM

Types of two solvents; one immobilized in the pores of the hollow fiber and the other located in the fiber lumen, were essential considerations in the HF-LPME for efficient extraction. First, the SLM must be compatible with the fiber so that the pores in the fiber wall can be filled completely and form a very thin organic membrane film. Second, the SLM must be immiscible with water and the acceptor organic phase. Finally, the acceptor organic solvent should have excellent chromatographic behavior [23]. According to the above points, among *n*-dodecane, *n*-nonane and 5% TOPO in *n*-dodecane, 5% TOPO in *n*-dodecane was selected as the SLM (Fig. 1). Here, TOPO increased the polarity of *n*-dodecane and improved the extraction of polar statin drugs. Alkaline acetonitrile, methanol, and water were evaluated in the subsequent experiments as the acceptor organic solvents. All the solvents showed excellent chromatographic behaviors, low solubility in *n*-dodecane, and effectively remained during the extraction (no leakage to SLM and no solvent loss due to evaporation). According to the results obtained, alkaline methanol, as the acceptor solvent, led to the best results. This means that mass transfer of the lipophilic drugs (log p > 4.2) from the organic membrane to the immiscible organic acceptor phase was higher than that to the aqueous acceptor. Thus, methanol was selected as the immiscible organic solvent for the acceptor phase.

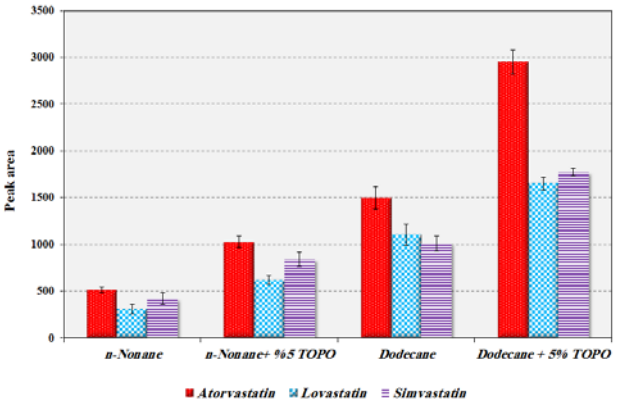

Fig. 1. Effect of SLM composition on extraction efficiency of atorvastatin, lovastatin, and simvastatin by HF-LPME; spiked concentration: 0.1 mg L^{-1}, sample solution: 0.01 mol L^{-1} HNO_3, acceptor solution: 0.01 mol L^{-1} NaOH in methanol, sample volume: 18 mL, extraction time: 30 min, and stirring rate: 600 rpm.

3.1.2. Selection of pH

Considering the acidic constants (pK_a) (Fig. 2), the three statin drugs in the current study are acidic. Therefore, changing the pH value could change their existing forms (neutral molecular forms or ion forms). In the donor phase, the statins should be in their uncharged form, so that they will be retained in the organic membrane phase when exposed to it. Appropriate amounts of a strong acid (0.1 mol L^{-1} HNO_3) were added to the donor phase to yield pH values of 1.0, 2.0, 3.0, 4.0, and 5.0. The *ER*s of atorvastatin, simvastatin, and lovastatin were the highest at pH 2.0 (Fig. 3). In order to retain the statin drugs in the acceptor phase and prevent diffusion back to the SLM, the compounds should be in their charged state once in the acceptor phase. Appropriate amounts of a strong base (1 mol L^{-1} NaOH) were added to methanol to achieve the desired alkaline levels. Therefore, solutions of 0.006, 0.01, 0.02, 0.05, and 0.1 mol L^{-1} NaOH in methanol applied as the acceptor phase. The NaOH solution (0.05 mol L^{-1}) in methanol was selected as the best acceptor phase.

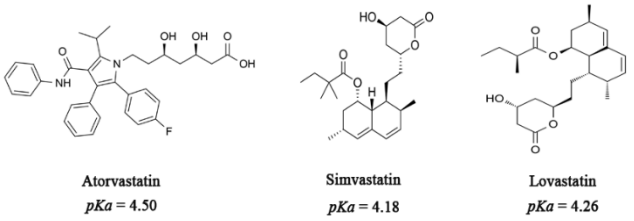

Fig. 2. Structures and pK_a of atorvastatin, simvastatin, and lovastatin.

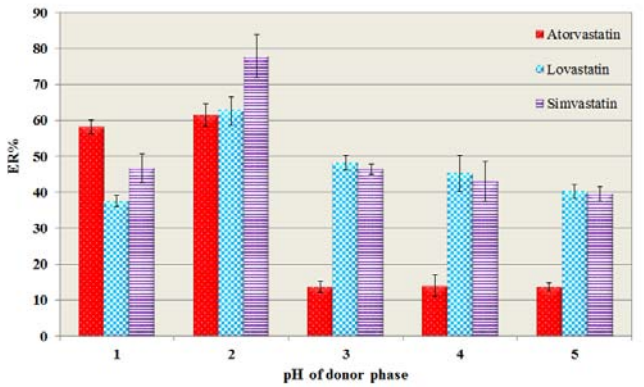

Fig. 3. Effect of initial pH of sample solution on extraction efficiency of atorvastatin, lovastatin, and simvastatin by HF-LPME; spiked concentration: 0.1 mg L^{-1}, SLM composition: 5% TOPO in *n* -dodecane, acceptor solution: 0.01 mol L^{-1} NaOH in methanol, sample volume: 18 mL, extraction time: 30 min, and stirring rate: 600 rpm.

3.1.3. Results for the central composite design

In this step, the values of other effective variables were optimized to obtain the best responses. In statistics, CCD is one of the most frequently used response surface designs [24].

In order to optimize the parameters that simultaneously influence the determination of statins, FCCCD, which is considered to be 1 in α (star point), was employed in the current study. The remaining effective factors; HF length (*A*), time of extraction (*B*), stirring rate (*C*), and salt % in the sample solution (*D*) were considered to maximize the experimental response (normalized peak area). Based on Eq. 4 with 4-factor and 6-center points totally 30 experiments had to be run for the FCCCD (Table 1).

Table 1. Effective factors, levels, and matrix of the face-centered central composite design (FCCCD)

Factors	Levels		
	-1	0	+1
A	4	7	10
B	10	35	60
C	100	550	1000
D	0	5	10

Runs	A	B	C	D	Normalized area
1	-1.0	1.0	1.0	-1.0	60.4
2[a]	0.0	0.0	0.0	0.0	103.5
3	1.0	-1.0	-1.0	1.0	20.1
4	1.0	-1.0	-1.0	-1.0	6.8
5[a]	0.0	0.0	0.0	0.0	106.9
6	-1.0	-1.0	1.0	-1.0	53.2
7	0.0	0.0	1.0	0.0	192.7
8	-1.0	-1.0	1.0	1.0	22.9
9	0.0	0.0	0.0	-1.0	165.1
10	0.0	-1.0	0.0	0.0	80.0
11	-1.0	-1.0	-1.0	1.0	9.2
12	0.0	1.0	0.0	0.0	126.5
13	0.0	0.0	0.0	1.0	45.2
14	1.0	1.0	1.0	-1.0	287.8
15	-1.0	1.0	-1.0	1.0	20.0
16	1.0	1.0	-1.0	-1.0	3.0
17	-1.0	1.0	1.0	1.0	53.9
18	-1.0	-1.0	-1.0	-1.0	27.8
19	1.0	0.0	0.0	0.0	109.3
20	-1.0	0.0	0.0	0.0	87.9
21	1.0	-1.0	1.0	1.0	56.3
22	0.0	0.0	-1.0	0.0	54.9
23[a]	0.0	0.0	0.0	0.0	106.0
24[a]	0.0	0.0	0.0	0.0	65.5
25	-1.0	1.0	-1.0	-1.0	91.6
26	1.0	1.0	-1.0	1.0	47.5
27	1.0	1.0	1.0	1.0	101.0
28	1.0	-1.0	1.0	-1.0	108.8
29[a]	0.0	0.0	0.0	0.0	106.0
30[a]	0.0	0.0	0.0	0.0	105.6

[a] Replications in center point.

The main effects were visualized by a Pareto chart (Fig. 4a). In this chart, the bar lengths are proportional to the absolute value of the main effects estimated. Fig. 4a also includes a vertical line corresponding

to 95% confidence interval. An effect, which exceeds this reference line, may be considered significant when the response is regarded. A positive value for the estimated effect indicates an increase in the response if the variable increases to its high level. A negative value indicates that a better response is obtained at low levels of the variable. For the interactions, a positive value indicates that the response will increase if both variables change to the same level, low or high. A negative value indicates an increase in the response if the variables change in the opposite directions (one variable increases to a high level and the other decreases to a low level). This chart implies that the factors of stirring rate, salt % in the sample solution, extraction time, and the interactions between HF length and stirring rate display statistically significant effects at the $P < 0.05$ level. The goodness of fit of the model can be checked by the coefficient of determination (R^2). Based on Joglekar and May [12], the least value of R^2 must be 0.8 (for the acceptable accordance of the model). The lack of fit is not significant relative to the pure error and the R^2 value (0.803) indicates a good relationship between the experimental and predicted values of the response. Eq. 5 illustrates the relationship between the four variables (i.e., HF length (*A*), extraction time (*B*), stirring rate (*C*), and salt% in the sample solution (*D*)) on the one hand and the normalized peak area on the other hand.

$$\textit{Normalized peak area} = 106.80 + 17.70A + 26.20B + 36.73C - 21.85D - 9.28A^2 + 8.72A.B + 27.48A.C - 3.09A.D - 34.6307B^2 + 10.53B.C - 7.96B.D + 15.91C^2 - 14.92C.D - 17.73D^2 \quad (5)$$

Also, ANOVA was performed and showed that the model was significant and the lack of fit was not significant ($P = 0.05$), which implied that the model was fitted (Table 2). As can be seen in Eq. 5, the stirring rate (*C*) and extraction time (*B*) have the largest influence on the extraction efficiency. The next most significant factors were quadratic terms of extraction time (*B*) and the linear term of salt% in the sample solution (*D*).

Based on the analyses and plots presented in Figs. 4b and 4c, the normalized peak areas of statins increase in a linear manner by increasing the extraction time, stirring rate, and length of HF to the given levels.

Table 2. ANOVA table for experimental responses obtained

Source	Sum of squares	Df	Mean square	F-ratio	P-value
A: HF length	5641.7	1	5641.7	3.82	0.0709
B: Time	12360.6	1	12360.6	8.37	0.0118
C: Stirring rate	24290.3	1	24290.3	16.46	0.0012
D: Salt%	8597.5	1	8597.5	5.82	0.0301
AA	222.7	1	222.7	0.15	0.7035
AB	1216.8	1	1216.8	0.82	0.3793
AC	12083.0	1	12083.0	8.19	0.0126
AD	153.5	1	153.5	0.10	0.7519
BB	3101.1	1	3101.1	2.10	0.1693
BC	1776.0	1	1775.0	1.20	0.2912
BD	1013.0	1	1013.0	0.69	0.4213
CC	655.3	1	655.3	0.44	0.5161
CD	3562.6	1	3562.6	2.41	0.1426
DD	812.9	1	812.9	0.55	0.4703
Lack of fit	19319.6	9	2146.6	7.97	
Pure error	1346.4	5	269.3		
Total error	20666.0	14	1476.1		
Total (cor.)	105160.0	28			

In this work, the influence of acceptor phase volume on the extraction efficiencies was studied using various hollow fiber lengths. In principle, in the three-phase HF-LPME methods, a smaller volume of the acceptor phase causes a higher analyte concentration in the acceptor phase. However, in the LPME, the total mass of the analyte in the acceptor phase is more important than the absolute concentration of the analyte [19]. Thus, the acceptor phase volume should be large enough to complete the analyte transport to the acceptor phase. Based on the obtained results, the peak area increased with an increase in the HF length. Therefore, the length of 10 cm was used in further experiments.

Moreover, the amounts of the analytes extracted increased dramatically by increasing the exposure time; and thereafter the curves became flat. More increase in the extraction time from these levels (45 min) leads to a decrease in the response (Fig. 4b). This phenomenon most probably was caused by analyte back-extraction

into the SLM due to saturation of the analyte in the acceptor phase; similar observation has previously been reported [25].

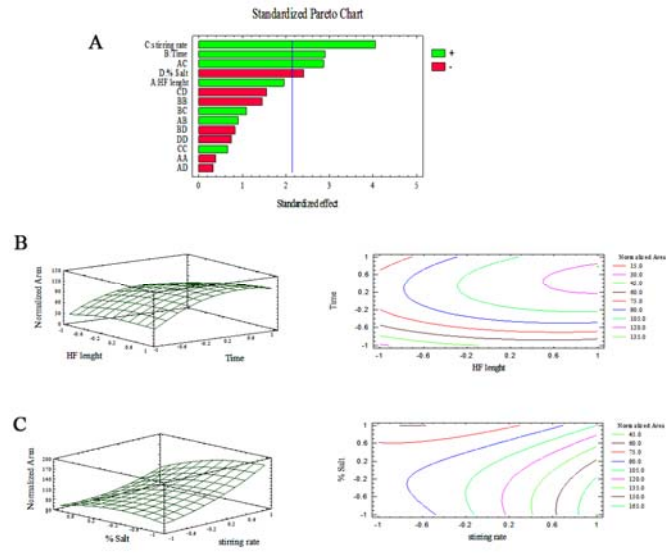

Fig. 4. (a) Pareto chart of the main effects for statins. (A) HF length, (B) extraction time, (C) stirring rate, (D) salt %; (b) response surface and contour plot of statins using FCCCD, which illustrates the relationship among the extraction time, HF length, and experimental responses in a three-dimensional representation; (c) response surface and contour plot of the statins using FCCCD, which illustrates the relationship between the stirring rate, salt%, and experimental responses in a three-dimensional representation

Taking into account the interaction between these variables, i.e. hollow fiber length and extraction time, the observed effect is positive; that is, by employing higher hollow fiber length, equilibrium was reached by employing higher extraction time. Stirring the solution facilitates the mass transfer process and reduces the time required to reach thermodynamic equilibrium. So, it has been universally used to improve microextraction efficiency [26]. In this work, stirring rate was optimized at the maximum magnetic stirrer performance, 1000 rpm (Fig. 4c).

Moreover, salt addition is a very useful way to enhance the extraction efficiency due to the salting out effect. Commonly, addition of salt can decrease the solubility of analytes in the aqueous sample and enhance their partitioning into the organic phase. Based on the obtained results (Fig. 4c), addition of salt did not improve the extraction efficiency. While the extraction efficiency was the highest without addition of sodium chloride it decreased subsequently as more salt was added. This may be related to formation of a physical barrier across the donor phase and the organic solvent interface, which could prevent the analyte mass transfer into the organic phase [26]. Therefore, the interaction between these variables, i.e., stirring rate and salt addition, the observed effect is negative. This means that by employing higher stirring rates, mass transfer of the analytes is favored through employing lower sodium chloride concentrations. Table 3 shows all the optimal conditions obtained, which were used for the rest of this work.

In order to decrease the matrix effect for extraction statin drugs from plasma samples methanol can be applied to release the analytes that bonded to proteins. Therefore, further experiments were conducted to investigate the effect of methanol addition on the extraction recoveries using variation of methanol volume (0.5, 1, 1.5, 2 mL) in pretreatment step. The results indicated that in the presence of 1 mL methanol maximum recoveries can be obtained. But the extraction recoveries decreased slightly in the presence of over 1 mL of methanol in the plasma solution due to increasing the solubility of the target compounds.

Method validation

The calibration curves were plotted in ultra-pure water and analyte-free urine and plasma samples under the optimal experimental conditions. All validation parameters such as limits of detection (LODs), limit of quantification (LOQ), linear dynamic ranges (LDRs), and intra- and inter-assay precision (RSD%) were obtained based guidelines of ICH of technical requirements for registration of pharmaceuticals for human use [27]. Analytical characteristics of the presented method (i.e., *PF*s, *ER*s and square of the correlation coefficients (R^2),) and validation parameters are displayed in Table 4.

Table 3. Optimal conditions for simultaneous extraction of some statins by HF-LPME.

Factors	Optimal condition
SLM	*n*-Dodecane + 5% TOPO
pH of donor phase	2
Acceptor phase	0.05 M NaOH in methanol
HF length	10 cm (containing 25 µL of acceptor phase)
Extraction time	45 min
Stirring speed	1000 rpm
Salt %	No salt

3.2.1. Performance of the presented method

To evaluate the linearity of the method, 15 spiking levels of atorvastatin, lovastatin, and simvastatin in the concentration range of 0.2 – 500 µg L^{-1} were used and the best LDR in the range of 0.5 – 400 µg L^{-1} and 1 – 500 µg L^{-1} was obtained for HPLC and GC, respectively. LOD for each statin was practically obtained based on a signal-to-noise ratio of 3. The *PFs*, which were calculated by Eq. 1, varied between 466 and 863 for HPLC and 550 up to 880 for GC. Some characteristics of previously reported methods such as extraction time, LODs, LDRs, and *PFs* for extraction and determination of statins are summarized in Table 5 for comparison. LODs and RSDs% in water, urine, and plasma samples were between 0.2 – 3 µg L^{-1} and 3.1 – 12.1% for HPLC and 0.2 – 5 µg L^{-1} and 2.2 – 6.8% for GC analysis, respectively. As can be seen, LODs, LDRs, RSDs%, and *PFs* of the current method are appropriate and comparable with those of the other methods reported for preconcentration and determination of statins.

3.2.2. Extraction of the statins from real samples

In order to investigate the practical applicability of the proposed extraction method for analysis of the drugs in real samples with complex matrices, the developed technique was applied for the target analyte analysis in the plasma and urine samples taken from patients that used some of these drugs. Due to high protein bonding of the drugs in the plasma (> 99%), the extraction recoveries of the plasma samples were low; so some pretreatments were required to eliminate the drug–protein interactions and also to release drugs from the plasma proteins. Therefore, to obtain higher analyte recoveries, the pretreatment was performed according to the following scheme: 1 mL of methanol was added to 2 mL of plasma with and without spiking of desired concentration levels of the drugs and the mixture obtained was strongly vortexed for 2 min. After placing the mixture on ice for 10 min, followed by 10 min at the ambient temperature, the mixture was centrifuged at 4000 rpm for 10 min. The supernatant was transferred into a 20-mL sample vial and diluted to the mark. Finally, the pH of the mixture was adjusted to 2.0 and the extraction procedure was followed under the optimal conditions. As a result, the matrix effect was reduced and high extraction recoveries were obtained. In order to reduce the matrix effects in the urine sample, it was diluted to 1:3 with ultrapure water. After dilution, pH of the sample was adjusted to 2.0. Then, the target analytes were extracted under the optimal conditions. Table 6 shows the best relative recoveries as 92 and 102% by HPLC-DAD and 89 and 95% by GC-FID instruments for the urine and plasma samples, respectively. Fig. 5 shows the chromatograms of HF-LPME extracts from the non-spiked and spiked urine with atorvastatin, lovastatin, and simvastatin under the optimal conditions (the spiked levels for the urine and plasma samples were 50 µg L^{-1} of the three statins). The results indicated that the proposed method has a high clean-up power and that the biological matrices had no significant effect on the extraction efficiency of the method.

Concluding remarks

In the present study, for the first time, the three-phase HF-LPME based two immiscible organic solvents was successfully developed for the extraction and preconcentration of three statin drugs in biological samples and analyzed by GC in addition to HPLC. Moreover, the experimental design method was efficiently employed to optimize the

Table 4. Figures of merit of the presented method

	Analyte	Matrix	*PF*	*ER%*	LOD (µg L^{-1})	LOQ (µg L^{-1})	Intra-day precision (RSD%, n = 3) RSD% (100 µg L^{-1})	Intra-day precision (RSD%, n = 3) RSD% (20 µg L^{-1})	Inter-day precision (RSD%, n = 3) RSD% (20 µg L^{-1})	Linearity LDR (µg L^{-1})	Linearity Regression equation	Linearity R^2
HF-LPME/HPLC-DAD	Atorvastatin	Water	863	106	0.2	0.5	4.7	6.9	6.6	0.5 – 400	A=68524C+102.12	0.998
		Urine	734	102	0.5	1	5.8	7.3	6.5	1 – 400	A=28953C+40.14	0.997
		Plasma	792	110	0.5	5	8.7	11.2	10.2	5 – 200	A=40390C+1352.90	0.992
	Lovastatin	Water	657	91.2	0.2	1	3.2	4.8	4.2	1 – 400	A=20235C+252.31	0.997
		Urine	466	64.7	3	5	3.6	5.6	4.6	5 – 400	A=10143C+22.71	0.999
		Plasma	576	80.0	0.5	5	7.5	10.8	8.0	5 – 200	A=14797C+101.35	0.982
	Simvastatin	Water	709	98.5	0.2	1	3.1	5.3	3.9	1 – 400	A=38954C-52.3	0.999
		Urine	507	70.4	3	5	3.7	6.4	4.4	5 – 400	A=13972C-94.29	0.998
		Plasma	684	95.0	0.5	5	10.2	12.1	11.0	5 – 200	A=21547C+428.35	0.985
GC-FID	Atorvastatin	Water	880	104.4	0.2	1	4.1	4.2	5.4	1 – 500	A=28654C+121.31	0.999
		Urine	855	95.0	0.5	2	5.3	5.2	6.2	2 – 500	A= 242596C- 1695.9	0.995
		Plasma	878	97.5	2	10	2.2	6.7	2.8	10 – 500	A= 203611C- 2345.8	0.997
	Lovastatin	Water	771	85.7	0.5	5	3.5	2.8	4.9	5 – 500	A=12365C+51.65	0.999
		Urine	550	61.2	5	10	4.6	3.0	6.1	10 – 500	A = 10714C+ 137.23	0.999
		Plasma	684	76.0	2	10	4.4	6.1	6.2	10 – 500	A= 9401.1C+ 86.32	0.999
	Simvastatin	Water	868	96.5	1	5	5.6	3.2	5.5	5 – 400	A=11257C+54.25	0.998
		Urine	863	68.2	5	10	5.7	4.5	5.9	10 – 500	A = 9558C + 56.67	0.999
		Plasma	734	93.0	2	10	3.5	6.8	4.7	10 – 500	A= 7224.1C+ 90.52	0.998

Table 5. Comparison of the presented method with some methods reported for extraction and determination of statin drugs

Analyte[a]	Analytical technique[b]	Matrix	Extraction time (min)	LDR (µg L^{-1})	LOD (µg L^{-1})	*ER%*	RSD% (intra-day)	Ref.
Ator, Sim, Lov, Pra, Flu, Rosu	DLLME/HPLC-Q-TOF-MS	Pure water	–	–	–	17 – 92	3.0 – 9.7	[1]
Ator, Sim, Lov, Pra, Flu, Rosu	SBSE/HPLC-Q-TOF-MS	Pure water	–	–	–	19 – 38	3.4 – 6.0	[1]
Ator, Sim, Lov, Pra, Flu, Rosu	SPE-HPLC/Q-TOF-MS	Pure water	–	–	–	74 – 93	1.1 – 6.9	[1]
Pita	SPE/HPLC-MS	Water	60	–	0.08	–	3.1 – 15.7	[9]
Ator	LLLME/HPLC-UV	plasma	–	1 – 500	0.4	91	4.4 – 7.7	[28]
Pra	LLE/HPLC-MS	Plasma	–	–	–	93 – 103	2.2 – 5.9	[29]
Ator, Lov, Sim	HF-LPME/HPLC-DAD	Water	45	0.5 – 400	0.2 – 3	64.7 – 110	3.1 – 12.1	This work
	HF-LPME/GC-FID	Urine Plasma	45	1– 500	0.2 – 5	61.2 – 104.4	2.2 – 6.8	

[a]Atorvastatin (Ator), Pitavastatin (Pita), Simvastatin (Simva), Lovastatin (Lov), Pravastatin (Pra), Fluvastatin (Flu), Rosuvastatin (rosu),
[b] Hollow fiber (HF), Liquid-phase microextraction (LPME), Liquid–liquid extraction (LLE), Liquid chromatography (LC), Mass spectrometry (MS), Solid-phase extraction (SPE), High-performance liquid chromatography (HPLC), Quadrupole-Time of flight (Q-TOF).

Table 6. Determination of statins in real samples

Analyte	Added concentration	*Urine* HPLC-DAD *Found*	*Urine* HPLC-DAD *RR%*	*Urine* GC-FID *Found*	*Urine* GC-FID *RR%*	*Plasma* HPLC-DAD *Found*	*Plasma* HPLC-DAD *RR%*	*Plasma* GC-FID *Found*	*Plasma* GC-FID *RR%*
Atorvastatin	0	17.0	–	15.5	–	25.0	–	23.2	–
	50	63.0	92	66.5	89	73.0	96	69.2	92
Lovastatin	0	ND [b]	–	ND	–	ND	–	ND	–
	50	41.0	82	37.5	75	49.0	98	45.0	90
Simvastatin	0	ND	–	ND	–	ND	–	ND	–
	50	45.0	90	42.5	85	51.0	102	47.5	95

[a] All concentrations are in µg L^{-1}.
[b] Not detected

HF-LPME conditions for analyzing the target drugs. A FCCCD was chosen to optimize the levels of the selected factors. The results demonstrated that the proposed method has good precision, linearity, and accuracy over the investigated concentration range. Also, the data herein represents the higher efficiency of HF-LPME based on two immiscible organic solvents compared to using an aqueous acceptor phase. This method has several advantages over other extraction methods:

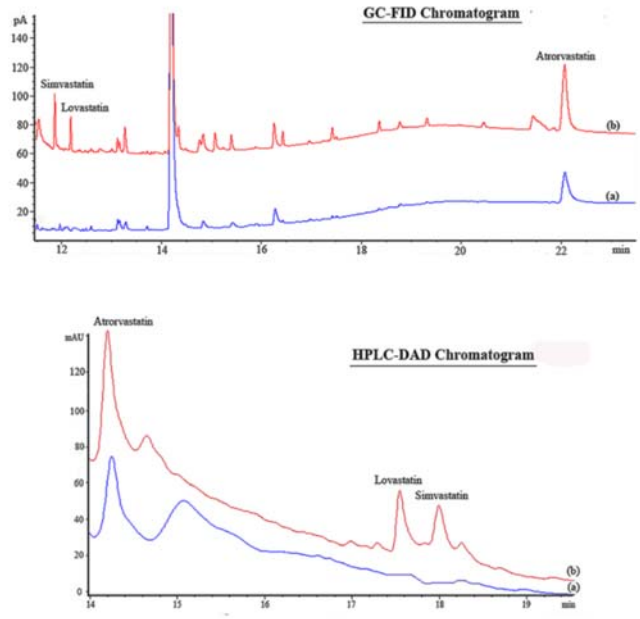

Fig. 5. The HPLC and GC chromatograms of the diluted urine sample (1:3) for (a) non-spiked and (b) 50 µg L-1 spiked of three satins under the optimal conditions (the sample solution pH, 2.0; stirring rate, 1000 rpm; HF length, 10 cm; and extraction time, 45 min).

(1) the equipment needed is very simple and inexpensive, (2) in the proposed three-phase mode, excellent clean-up has been observed, even for complicated urine and plasma samples, (3) because of high extraction recovery obtained (> 61.2 %), the proposed three-phase LPME provided very high preconcentration factor (466 - 878); thus no further concentration of the extract is required before the final analysis, (4) due to using organic solvent as acceptor phase, after the extraction, the extract is directly injected into the GC, (5) due to simplicity and the low cost of the extraction device, the hollow fiber can be discarded after each extraction to eliminate possible carry-over problems and cross-contaminations as compared to the SPME. This maintains high reproducibility and repeatability for the method, (6) the organic phase volume is less than 0.03 mL, resulting in an extremely low consumption of organic solvent per extraction. Thus, the present HF-LPME method may therefore be utilized as a green chemistry approach to reduce the consumption of hazardous organic solvents in chemical laboratories, (7) although the extraction time was relatively long (45 min), by applying a multi-stirrer, many samples could be extracted simultaneously, and finally (8) HF-LPME method especially based on two immiscible organic solvents was applied for the first time to extract some statin drugs and analyzed by GC.

Notes and references

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Three-phase hollow fiber microextraction based on two immiscible organic solvents for extraction and preconcentration of three statins in biological samples