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Research Article

Microextraction of Furosemide from Human Serum and Its Fluorimetric Determination

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ABSTRACT

Background: A new, fast and sensitive spectrofluorimetric method was proposed for the determination of furosemide in serum samples based on a dispersive liquid-liquid microextraction.
Methods: The optimum conditions for quantification of furosemide were studied considering the effects of types and amounts of dispersive and extraction solvents, salt addition, pH value, rate and duration of centrifugation. The method was validated with respect to the linearity, recovery and limit of detection.
***Results*:** Under the optimal conditions, the fluorescence intensities at 406 nm (with the excitation wavelength of 342 nm) were linear with the concentration of furosemide in the range of 0.3 to 20 µg mL^{-1}, with a detection limit of 0.12 µg mL^{-1} and a relative standard deviation of 3.4–9.4%.
Conclusion: Careful examination of the obtained validation results reveal that the proposed method is suitable for determination of furosemide in serum samples.

Introduction

Furosemide (FUR) is a potent and short-acting diuretic[1] used for the treatment of cardiac, hepatic or renal originated oedema. It is prescribed with therapeutic dose range of 40 to 200 mg daily in adults.[1] Treatment of oedema could be achieved with an initial oral dose of 40 mg daily; however in severe cases, up to 600 mg daily may be required. In acute or chronic failure patients, up to 6 g is given as slow intravenous infusions.[2] Quantification of FUR is required in many biomedical/pharmaceutical applications including in therapeutic drug monitoring. Due to increasing urine volume and masking the other doping agents, FUR is listed in World Anti-Doping Agency`s banned drugs.[3] Therefore, determination of FUR in biological fluids by a simple, sensitive and inexpensive method is an important task.

Several analytical methods have been reported for determination of FUR such as spectroscopy,[4-8] chromatography,[9-21] and voltammetry.[22,23] Also, FUR has been determined by spectrofluorimetry, using solid phase extraction (SPE) for preconcentration.[24,25]

Dispersive liquid-liquid microextraction (DLLME) method have been used in many biomedical analytical methods and possesses several advantages such as simplicity, low sample volume, low cost and high efficiency.[26,27] Various analytes such as barbituric acid,[28] losartan and carvedilol,[29] cyproheptadine,[30] valproic acid,[31,32] terazosin,[33] carvedilol,[34,35] bosentan,[36] metoprolol, propranolol, carvedilol, verapamil, diltiazem,[37,38] verapamil,[39] methadone,[40] furosemide,[41] and aluminium,[42] have been extracted from biological samples by DLLME and quantified using different analytical methods.

The aim of this study was to present a sensitive and simple method for the determination of FUR in serum samples based on DLLME-spectrofluorimetry. The proposed method was validated according to FDA guidelines and applied on a limited number of real serum samples.

Materials and Methods

All chemicals and solvents were of analytical reagent grade. Deionized water from Shahid Ghazi Pharmaceutical Company (Tabriz, Iran) was used throughout. Acetonitrile, chloroform, hydrochloric acid (HCl), methanol, NaCl were obtained from Merck (Darmstadt, Germany) and furosemide powder from Alborz Darou Company (Tehran, Iran). A stock solution (1000 µg mL^{-1}) of furosemide was prepared by dissolving the appropriate amount of drug in methanol (due to its low aqueous solubility)[43] and stored in a refrigerator.

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Apparatus

Fluorescence spectra and intensity measurements were performed by means of a Jasco FP-750 spectrofluorimeter (Kyoto, Japan) equipped with a 150 W xenon lamp, using a 100 µL quartz microcell (Starna, UK). The excitation and emission monochromator bandwidths were 5 nm. The excitation wavelength was set at 342 nm and the fluorescence was measured at 406 nm. All measurements were performed at 25 °C, controlled using a Peltier thermostated cell holder (Jasco, Japan). The pH of solutions was measured with Metrohm 654 pH meter (Herisau, Switzerland). A Hettich (EBA-20) centrifuge (Germany) and a Labtron (LS-100) vortex shaker (Iran) were used for centrifuging and shaking the solutions, respectively.

Sample preparation

Serum samples of healthy donors were obtained from Tabriz Blood Transfusion Organization and spiked with appropriate concentrations of FUR in which the final concentrations were in the range of 0.3-20 µg mL^{-1}. Four healthy volunteers were taken a single dose of 40 mg of FUR and then 5 mL of their blood samples were collected for separating their serum. The sample donors signed a written consent form approved by ethics committee of Tabriz University of Medical Sciences.

General procedure.

After precipitation of serum proteins by addition of 1:1 ratio of acetonitrile, shaking and centrifuging, 0.8 mL of serum sample containing FUR were placed into a 10 mL glass test tube with conical bottom and diluted to 8.0 mL and NaCl 6% (w/v) and convenient volume of HCl (0.1M) solution was added to adjust the pH at 2.0.

Methanol (700 µL, as dispersive solvent) was mixed with chloroform (200 µL, as extraction solvent) and rapidly injected into the above mentioned sample. After gentle shaking, a cloudy solution was formed and FUR from aqueous samples were extracted into the droplets of chloroform. The mixture was centrifuged for 7.0 min at 5000 rpm. The dispersed fine particles of organic phase were sedimented in the bottom of conical test tube. 100.0 µL of sedimented phase was transferred into the microcell and its fluorescence intensity was recorded at 406 nm.

Results and Discussion

Selection of extraction solvent

Several factors such as higher density in comparison with water, low solubility in water and high extraction capability of interested compounds should be considered for selection of extraction solvent.[44] Chloroform, dichloromethane, carbon tetrachloride and 1,2-dichlorobenzol were studied in this work. All optimization experiments were carried out using serum samples spiked with solution of FUR (6 µg mL^{-1}). Based on the results, illustrated in (Figure 1 (a)), chloroform was chosen as the extraction solvent.

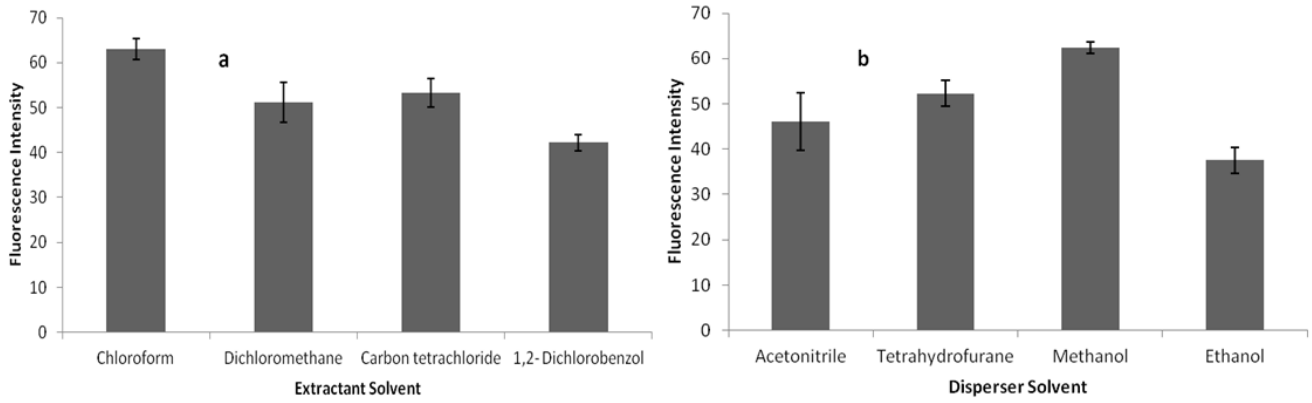

Figure 1. Effect of type of extraction solvent (a) and disperser solvent (b) on the efficiency of microextraction of FUR.

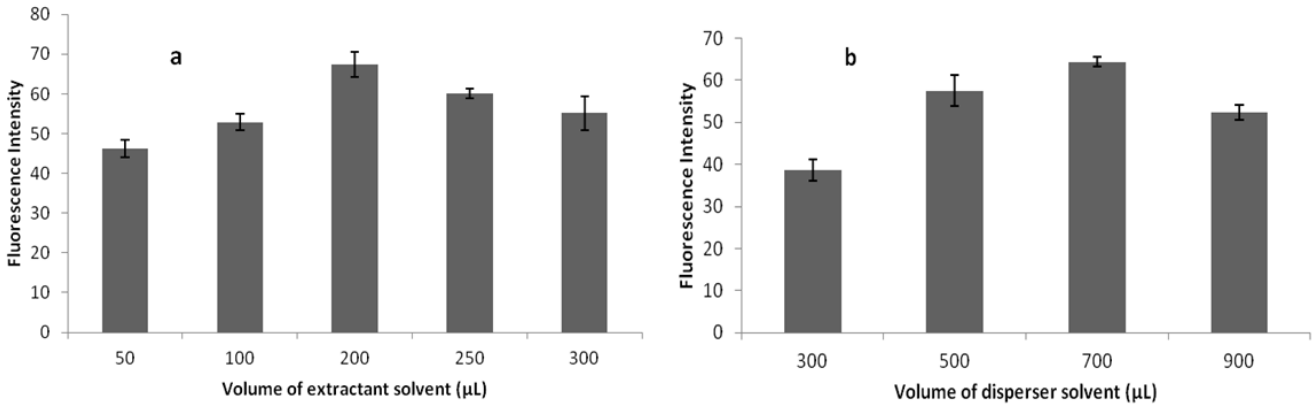

Figure 2. Effect of extraction solvent volume (a), and disperser solvent volume (b) on the DLLME of FUR.

Selection of disperser solvent

The miscibility with the organic and aqueous phases is the most important characteristics of a dispersive solvent.[45] It helps to produce fine droplets of extraction solvent and its distribution throughout the aqueous solution and finally increases the contact area with the sample solution. The disperser solvent was selected among acetonitrile, tetrahydrofuran, methanol and ethanol. The obtained results shown in (Figure 1(b)), revealed that methanol is the best disperser solvent.

Effect of extraction and disperser solvents volume

Volume of extraction solvent affects the extraction efficiency of analyte. On the other hand, the amount of disperser solvent influences the droplet formation. At low volumes of disperser solvent, fine droplets of extraction solvent and cloudy solution are not formed and so, the extraction efficiency is reduced. At the higher volume of disperser solvent, solubility of the analyte in solution increases and the distribution of the analyte in the extraction solvent is reduced, resulting in decreased extraction efficiency. According to the above description, effects of both extraction and disperser solvents volumes were studied.

Volume of extraction solvent was studied by using 50, 100, 200, 250 and 300 µL of chloroform, in the presence of 700 µL of acetonitrile. According to the results (Figure 2(a)) 200 µL of chloroform, was selected as the appropriate volume. Using this amount, the volume of methanol differed from 300 µL to 900 µL was studied. Based on the results illustrated in (Figure 2(b)), 700 µL of methanol was selected as the optimum volume of disperser solvent.

Effect of pH

In sample solutions, analyte should be in neutral form in order to be able to transfer to the organic phase (extraction solvent) and this is dependent on the pH of the solution. In other words, the charged analytes cannot be solved in the organic phase, and this indicate that extraction of ionized analytes including; polar drugs, metal ions etc. is significantly affected by the pH of the sample.[46] Therefore, the effect of pH on FUR extraction was studied. Based on results shown in (Figure 3), pH=2.0 was selected because of higher fluorescence intensity and better repeatability.

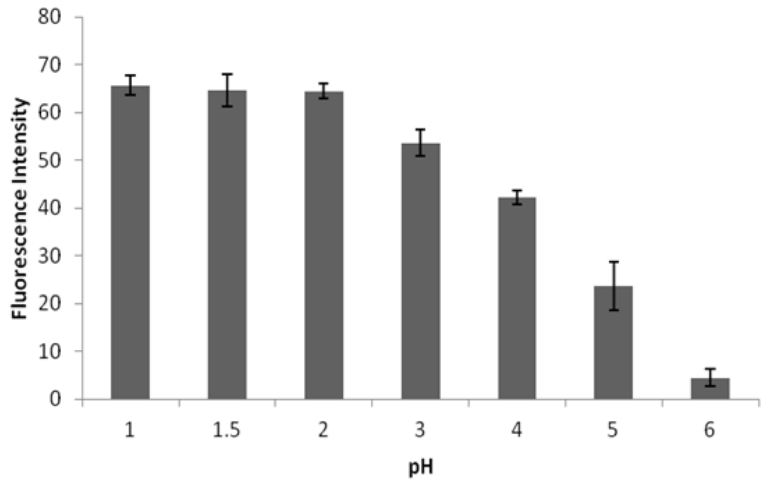

Figure 3. Effect of pH on the DLLME of FUR.

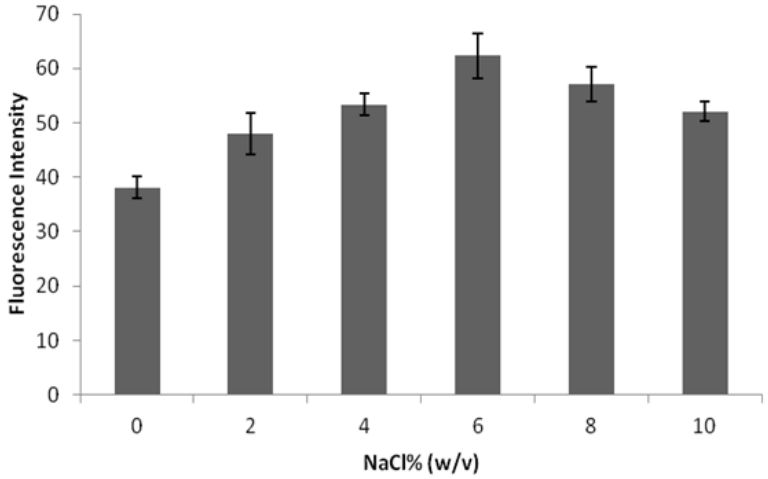

Figure 4. Effect of salt addition on the DLLME of FUR.

Table 1. Comparison of linear range (LR) and limit of detection (LOD) for determination of FUR in blood or its derivative samples.

Method	LR (µg mL^{-1})	LOD (µg mL^{-1})	Sample	Reference
HPLC-MS-MS	0.02-1.6	-	Human plasma	12
HPLC	0.25-5	0.01	Serum	20
RP-HPLC	0.5-10	-	Human serum	21
capillary isotachophoresis	0.05-1.0	0.03	Serum	50
GC-EI-MS	0.1-5	0.01	Whole blood	51
DLLME-Spectrofluorimetry	0.3-20	0.12	Human serum	This work

Effect of salt addition

Concerning the addition of salt in liquid phase microextraction methods, conflicting results have been reported.[47] Sometimes a reduction in extraction efficiency at higher salt concentrations has been reported, which is due to the increase in viscosity of aqueous solution, that could limit the transfer of analytes into the extraction solvent.[48] On the other hand, due to the "salting-out" effect, solubility of many analytes in aqueous solutions decreases with increasing ionic strength of solution.

As a result of this effect, aqueous solvent molecules started to hydrate the ions generated from salt and hydrated analyte molecules are released and the analyte solubility in the aqueous phase decreases. Therefore, use of lower volumes of extraction solvent is possible.[49] Effect of added salt on FUR extraction was studied by adding various concentrations of NaCl to the spiked serum samples. As can be seen in (Figure 4), in the presence of 6% (w/v) NaCl, highest extraction efficiency was obtained.

Centrifuging rate and time

Effect of time and rate of centrifugation was also studied. Based on results, the optimum rate and time for centrifuging were 5000 rpm and 7 min, respectively.

Analytical figures of merit

The method was validated according to FDA guideline and the entire process was carried out in the serum matrix. In the optimum conditions for extraction of FUR, different solutions of FUR with 0.3-20 µg mL^{-1} concentrations were prepared in serum samples and their fluorescence intensities were recorded.

The method was linear in the range of 0.3 to 20 µg mL^{-1}, with a correlation coefficient of 0.992. The limit of detection (LOD), lower limit of quantification and upper limit of quantification were 0.12, 0.3 and 20 µg mL^{-1}, respectively. A comparison between the LODs, linear range, and sample types obtained in this work and those from other reported methods was listed in (Table 1).

Precision of method was calculated by both inter-day and intraday experiments. In inter-day experiment, three spiked solution of FUR with 4.0, 12.0 and 18.0 µg.mL^{-1} concentrations were prepared in serum and analyzed by presented method during a specific day (n=3). Similarly, for intra-day experiment, spiked solutions of FUR with same concentrations were analyzed at three consecutive days (n=3).

Accuracy of the proposed method was determined by calculating the recovery percentages in three spiked samples. Experiments were carried out in three different days and were repeated for three times for each concentration. The results for precision, accuracy and recovery of the proposed method are given in (Table 2), which results illustrate that the proposed method is accurate and precise for FUR determination in serum sample.

Table 2. Percent recovery and relative standard deviation (RSD) of spiked serum samples.

Concentration (µg.mL^{-1})	Inter-day precision (%RSD)	Intra-day precision (%RSD)	Recovery (%)
4.0	8.2	9.4	98.8
12.0	6.3	7.2	102.3
18.0	3.9	3.4	100.1

Short term temperature and freeze/thaw stabilities were studied for the proposed method. Short term temperature stability of method was studied using three spiked solutions of FUR in serum, containing 4.0, 12.0 and 18.0 µg mL^{-1} concentrations. These samples were kept at room temperature for 8 h and then concentration of FUR was determined. Same solutions prepared, were kept at room temperature for 10 h, then frozen for 10 h and kept again at room temperature for 10 h. After this procedure, concentration of FUR was determined. The stability data for the proposed method is listed in (Table 3), and the results indicate that FUR is stable in the investigated conditions.

Table 3. Results for study of stability of the method.

Concentration (µg.mL^{-1})	%Recovery for short term temperature stability	%Recovery for freeze and thaw stability
4.0	101.75	100.50
12.0	97.66	101.41
18.0	104.05	98.94

Table 4. Influence of interfering of some cardiac drugs on the determination 0.5 µg mL^{-1} furosemide.

Interfering agent	Concentration of interfering agent (µg mL^{-1})	Variation in fluorescence intensity (%ΔF)
Atorvastatin	450	-4.5
Captopril	500	3.2
Digoxin	400	5.1
Enalapril	500	-1.2
Losartan	350	-2.0
Lovastatin	250	3.5
Nitroglycerin	250	2.4
Oxazepam	500	4.5
Pantoprazole	500	4.2
Clopidogrel	250	-2.0
Spironolactone	500	-3.2
Valsartan	500	2.8

Table 5. The obtained analytical conditions for analysis of furosemide in serum and urine[41] samples.

Sample Analytical condition	Serum	Urine
Extraction solvent (volume)	Chloroform (200 μL)	Chloroform (250 μL)
Disperser solvent (volume)	Methanol (700 μL)	Acetonitrile (600 μL)
pH	2.0	2.0
Salt addition	6 % w/v	10 % w/v
Centrifugation rate (and time)	5000 rpm (7 min)	4000 rpm (7 min)

Study of interferences

Variation of spectrofluorimetric response of FUR was followed in the presence of some other drugs. Different concentrations (250-500 μg.mL^{-1}) of some cardiac drugs were added to serum samples containing 0.5 μg mL^{-1} of FUR. Spectrofluorimetric responses of these solutions were compared with that obtained from FUR with the same concentration, extracted by DLLME in the absence of other drugs and the results were shown in (Table 4). Based on these results, it can be concluded that the method has good selectivity for determination of FUR.

Comparison of the obtained analytical conditions for urine and serum samples

As noticed in Introduction, a similar method was developed and validated for quantification of FUR in urine samples.[41] Table 5 reports a brief summary of the obtained analytical conditions for urine[41] and serum samples. Chloroform was the extraction solvent for both samples, for serum, methanol (700 μL) was best acted as disperser solvent whereas for urine acetonitrile (600 μL) was the best disperser solvent. Salt addition was improved the extraction performance with different amounts for serum (6 % w/v) and urine samples (10 % w/v) possibly due to lower concentration of NaCl in urine samples. The centrifugation rates were slightly different from serum and urine samples, because of lower viscosity of urine samples.

Analysis of real Samples

Ability of the method for determination of FUR in real samples was tested. For this purpose, sampling performed from four healthy volunteers who received a 40 mg dose of FUR. Blood samplings were carried out 2 h after FUR intake and their serums were separated. Samples were treated by DLLME and amount of FUR were determined using spectrofluorimetery (Table 6). Considering the serum concentration of FUR (1.8-4.9 μg·mL^{-1}),[51] the results listed in Table 5 prove the ability of the presented method for determination of FUR in complex biological matrices.

Table 6. Analysis of real samples by the proposed method.

Volunteer	Gender	Age (year)	Concentration ± SD(μg.mL^{-1})
1	M	25	2.8 ± 0. 5
2	M	26	1.7 ± 0.3
3	M	27	1.2 ± 0.4
4	M	27	2.1 ± 0.3

Conclusion

A sensitive, simple and fast spectrofluorimetric method was developed and validated for determination of FUR in human serum after a dispersive liquid-liquid microextraction. The proposed method is an accurate, fast and precise procedure for the determination of FUR and no interference from other cardiovascular drugs was observed. This method also exhibits significant stability for determination of FUR in serum.

Conflict of interests

The authors claim that there is no conflict of interest.

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