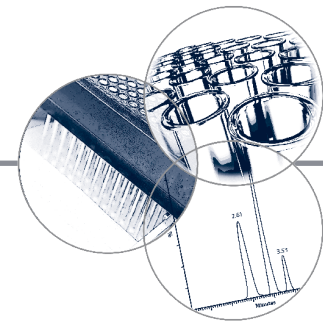
RESEARCH ARTICLE



Dispersive liquid–liquid microextraction based on solidification of floating organic droplet followed by spectrofluorimetry for determination of carvedilol in human plasma

Background: Simple, chip and rapid analytical methods are required in biomedical analysis laboratories to support therapeutic drug monitoring units in hospitals. The present work aimed to provide such a method for quantitative determination of carvedilol in plasma samples. **Results:** A new, simple, precise and efficient method was developed for the determination of carvedilol in human plasma using a dispersive liquid–liquid microextraction based on solidification of floating organic droplet, followed by spectrofluorimetry method. Some important parameters such as types and volumes of extraction and disperser solvents, pH, salt effect and sample volume were optimized. Under the optimized experimental conditions, the method provided a linear range of 40 to 300 ng ml^{-1}, with a correlation coefficient of 0.996. The limit of detection, lower limit of quantification and upper limit of quantification were 18, 40 and 300 ng ml^{-1}, respectively. The found recovery was from 98.2 to 102.2%, the mean intra- and inter-day precisions were 8.3 and 6.4%, respectively. The relative error for accuracy varied from 0.4 to 2.2%. The short-term temperature and freeze–thaw stability studies showed that carvedilol in human plasma was stable for sample preparation and analysis after storage. **Conclusion:** The proposed method provided reasonable acceptable results and could be used for therapeutic monitoring of carvedilol.

Carvedilol or 1-(9H-carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy) ethyl] amino]-2-propanol (Figure 1) [1], is a nonselective β-adrenergic blocking agent with α_1-blocking activity [2], which also has vasodilating properties that are mainly attributed to its blocking activity at α_1 receptors [3] and anti-proliferative and antioxidative properties [4]. Carvedilol is used for the treatment of hypertension, angina pectoris and mild or moderate heart failure of ischemic or cardiomyopathic origin and can mitigate tardive movement disorders, psychosis, mania and depression [5], and is also sometimes abused by athletes for performance enhancement. Carvedilol is classified as a prohibited drug by the World Anti-Doping Agency [6] and the International Olympic Committee.

The therapeutic concentration of carvedilol in plasma is in the range of 0.02 to 0.16 µg ml^{-1} [7]. It should be noted that most patients are taking moderate-to-high doses of carvedilol and the plasma concentration of drug will be at the median (or above) of its therapeutic range. Carvedilol is rapidly and well absorbed after oral administration, but is subjected to considerable first-pass metabolism in the liver; and its absolute bioavailability is about 25%. Peak plasma concentrations occur 1 to 2 h after administration and it is highly protein bound (>98%) [8,9]. Relative to other β-blockers, carvedilol has minimal inverse agonist activity and it provides additional morbidity and mortality benefits in congestive heart failure [2].

Different analytical techniques have been employed for the determination of carvedilol either in pharmaceutical preparations or in biological samples. These methods include; spectrophotometry [10], chemiluminescence [11], electrochemical [12], GC [13], HPLC [1,14–22] and capillary electrophoresis [23,24] methods. Also, study of the fluorescence property of carvedilol and its tablet formulations by fluorimetry [25] was reported. Some of the details of the used methods for the determination of carvedilol are given in Table 1. Fluorescence spectrometry is widely used in quantitative analysis because of its great sensitivity and selectivity as well as its relatively low cost [26]. For determination of traces of drugs in plasma, the development of a simple, rapid, selective and sensitive spectrofluorimetric method is highly desirable. In particular, it is suitable for the determination

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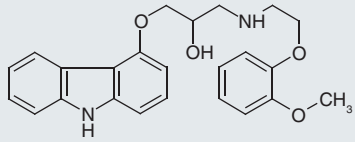

Figure 1. Carvedilol.

of carvedilol because it presents natural fluorescence. Many analytical techniques; including spectrofluorimetric methods, cannot be directly applied to complex matrices due to the presence of fluorescence interferences in these matrices. There is another problem in the spectrofluorimetric determination of drugs in biological matrices, that is, the significant analyte–background interactions that may lead to quenching of the analyte signal by blood or urine proteins [27]. A plasma sample is the most complex and commonly analyzed biological matrix. Proteins (e.g., albumin, α_1-acid glycoprotein and lipoproteins) exist in this matrix at high concentrations [28]. Therefore, prior to its analysis, utilization of a technique to remove these interferences is necessary, especially for trace analysis of the analytes.

Sample preparation is an important step in the determination of trace amount of analytes in complex sample matrices such as plasma and influences their reliable and accurate analysis. The aims of sample preparation could be extraction of the analytes (which results in the isolation of analytes from interferences of matrices) or preconcentration of analytes from the sample matrix to improve performance of the analytical method. It is necessary and important to **clean up** the samples with difficult and complex matrices. Recent progresses in sample preparation have been focused on miniaturization, automation, low operation costs and reduction in solvent consumption and operation time. Liquid-phase microextraction (LPME) is a sample preparation procedure that uses minimized amount of solvents. An innovation in LPME led to the invention of dispersive liquid–liquid microextraction (DLLME) by Assadi and coworkers in 2006 [29]. It consisted of two steps: first, mix appropriate extraction and disperser solvents and rapidly inject into an aqueous sample – a dispersion is formed throughout the aqueous sample and facilitates fast extraction of analytes from the aqueous sample; and second, the dispersion is removed by centrifugation and

Table 1. Some previous studies for determination of carvedilol in biological fluids.

Method	Sample	Sample preparation	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	Linear range (ng ml^{-1})	Ref.
HPLC–fluorimetry	Human plasma	Protein precipitation and concentration of the supernatant by evaporation	NR	1.3	1.303–142	[16]
HPLC–fluorimetry	Human plasma	Protein precipitation procedure	NR	1.0	1–80	[15]
HPLC–fluorimetry	Human plasma	LLE	NR	1.0	1–128	[1]
HPLC–fluorimetry	Rat plasma	SPE	3.6	NR	0–1000	[17]
HPLC–fluorimetry	Human plasma	Online sample preparation	NR	0.8	1–50	[18]
HPLC–UV	Human serum	LLE	2.5	5.0	5–500	[14]
HPLC–UV	Human serum	Stir bar sorptive extraction	0.3	1.0	1–120	[22]
HPLC ECD	Human plasma	–	NR	0.1	0.1–150	[19]
HPLC–MS/MS	Human plasma	LLE	NR	0.1	0.1–200	[20]
LC–MS/MS	Human plasma	SPE	NR	0.5	0.5–200	[21]
HILIC–MS/MS	Human plasma	LLE	NR	0.1	0.1–200	[38]
GC–MS	Human urine	LLE	0.3	0.75	0.75–75	[13]
CE–UV	Human serum	LLE	NR	50.0	50–4000	[24]
CE–UV	Human urine	SPE	50.0	80.0	50–500	[23]

ECD: Electrochemical detection; HILIC–MS/MS: Hydrophilic interaction LC with MS/MS; LLE: Liquid–liquid extraction; NR: Not reported.

Key Terms

Carvedilol: Antihypertensive drug from β-blocker groups widely used in therapeutics,

Clean up: Pretreatments on samples to eliminate the interfere analytes from the matrix .

two-phase separation occurs and the extraction solvent containing the analytes of interest is separated and is taken easily with a microsyringe [30]. It is a simple, fast, cheap and efficient microextraction technique with high enrichment factor and recovery; however, the number of extraction solvents are limited because of the need to use solvents with a higher density than water, which are collected at the bottom of centrifuge tube. The highly toxic extraction solvents used are chlorobenzene, chloroform and carbon tetrachloride.

To overcome these drawbacks, a DLLME technique based on solidification of floating organic droplets (DLLME-SFO) was developed in 2008 by Leong and Huang [31], which uses low-density and low melting point organic compounds as extraction solvents. This technique is similar to DLLME with additional cooling and solidification steps. The solidified floating phase is easily collected for analysis, which provides a high precision result to prevent the loss of analyte. The large contact surface between the sample and the droplets of extraction solvent in DLLME speeds up the mass transfer phenomenon. The use of extraction solvents with lower toxicity in this method is environmentally friendly and there is no need to use conical bottom glass tubes, which are easily damaged and hard to clean.

In the present work, the DLLME-SFO spectrofluorimetric method is developed for the determination of carvedilol in human plasma as a quick and easy-to-use method to achieve the plasma concentrations of carvedilol. The effects of various experimental parameters, such as types of extraction and disperser solvents, volumes of extraction and disperser solvents, pH, salt addition, time, speed of centrifugation and sample volume on the fluorescence intensity of carvedilol, and thus its extraction in spiked human plasma samples, were investigated. The proposed method was validated according to US FDA guidelines [32] and then successfully applied for the analysis of carvedilol in a number of real plasma samples.

Experimental

Apparatus

Fluorescence spectra and intensity measurements were performed on a Jasco FP-750 spectrofluorimeter (Tokyo, Japan) equipped with a 150 W xenon lamp, and using a 10 mm quartz cell. Excitation and emission slits of monochromators were both adjusted to 5 nm. The excitation wavelength was set at 286 nm and the fluorescence intensity was measured at 344 nm. All measurements were performed at 25°C, controlled using a Peltier thermostat cell holder from Jasco (Tokyo, Japan).

Chemicals & solutions

All chemicals used were of high analytical purity grade. Carvedilol powder was purchased from Salehan Shimi (Tehran, Iran). 1-undecanol, 1-dodecanol and sodium hydroxide were purchased from Merck (Darmstadt, Germany), acetonitrile (HPLC-grade), acetone (ultra-pure), methanol (ultra-pure), hydrochloric acid (37% HCl) and sodium chloride were obtained from Scharlau (Barcelona, Spain) and tetrahydrofuran was obtained from Romil SpS (Tehran, Iran). Deionized water was purchased from Ghazi Pharmaceutical Company (Tabriz, Iran). Stock standard solution of carvedilol (1000 µg ml^{-1}) was prepared in methanol and the daily standard working solutions of different concentrations were prepared by diluting the stock solution with methanol. All were stored at 4°C.

Collection & preparation of plasma sample

Drug-free plasma samples were obtained from the Iranian Blood Transfusion Research Center (Tabriz, Iran) and aliquots were transferred into polypropylene microtubes and frozen at -4°C until analysis. Drug-free plasma samples frozen at -4°C were thawed at room temperature on the day of extraction and were vortexed to ensure homogeneity. Subsequently, for preparation of desired concentration (200 ng ml^{-1}) of carvedilol in plasma, 14 µl of 10 µg ml^{-1} carvedilol standard solution was spiked into 700 µl drug-free plasma in a polypropylene microtube and was vortexed for 20 s and kept at room temperature for 20 min. Then, for precipitation of plasma proteins, acetone was added with 1:1 ratio and vortexed for 20 s. Following this, it was centrifuged for 10 min at 6000 rpm. Thus, the plasma proteins were precipitated and clear supernatant solution was transferred for use. After precipitation of proteins, 1.0 ml of clear supernatant solution was transferred in a 10.0 ml volumetric flask and water and 1.5 g NaCl were added. After dissolving NaCl, the pH of obtained solution was adjusted to 12.00 by NaOH 1 M. Thus, in this step, 1 ml of clear supernatant solution after precipitation of proteins was diluted to 10 ml by water for the microextraction procedure.

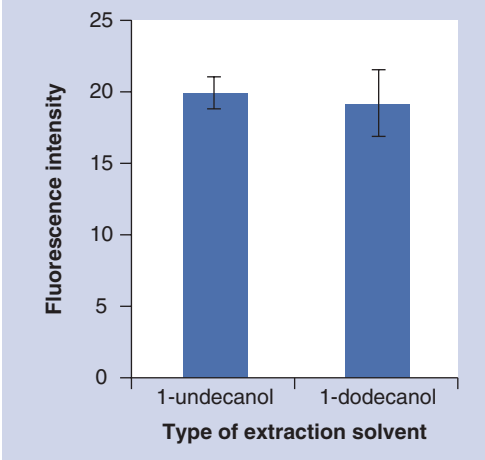

Figure 2. Optimization of extraction solvent. Concentration of spiked carvedilol in plasma: 200 ng ml^{-1}; extraction solvent volume: 100 µl; type and volume of disperser solvent: 1.0 ml acetonitrile; pH = 12.00; salt amount: 4.0% w/v NaCl; speed and time of centrifugation: 6000 rpm for 5 min; sample volume: 10 ml. λ_{ex} = 286 nm; λ_{em} = 344 nm; monochromators slits width: 5 nm; spectrofluorimeter temperature: 25°C.

DLLME-SFO procedure

In the next step, after diluting 1 ml plasma sample and preparation of the sample solution, 10.0 ml sample solution was placed in a 10.0 ml glass test tube. 75.0 µl of 1-undecanol (as an extraction solvent) and 0.50 ml of acetonitrile (as a disperser solvent) were mixed and the mixture was injected rapidly into the aqueous sample solution by a plastic syringe. A cloudy solution formed immediately and after centrifugation at 6000 rpm for 5 min, the organic solvent drop was floated on the surface of the aqueous sample solution due to its low density. Next, the glass test tube was cooled in ice for 10 min and, thus, the floated solvent was solidified due to its low melting point (13–15°C [33]). The solidified organic solvent was then easily transferred to a polypropylene microtube by a medical forceps and melted quickly at room temperature. Prior to analyzing by spectrofluorimetry, the extracted phase was mixed with methanol to 1.0 ml in a polypropylene microtube.

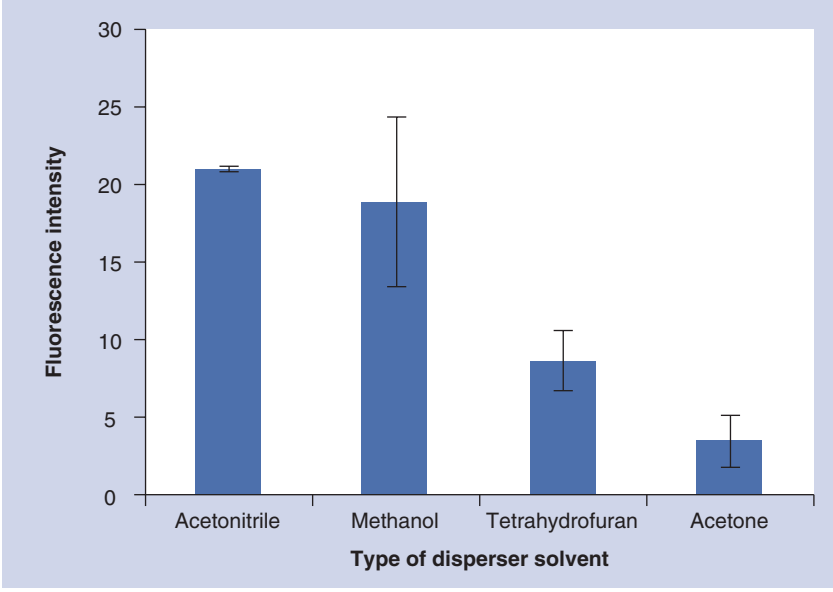

Figure 3. Effect of type of disperser solvent on extraction of carvedilol. Experimental conditions are the same as in Figure 2, and the optimized type of extraction solvent is 1-undecanol.

■ Preparation of real plasma samples

Blood samples were collected from patients who were receiving carvedilol at Madani Hospital. The patients were informed about the objectives of the project and signed a consent form approved by the ethical committee of Tabriz University of Medical Sciences. The blood samples were transferred to the heparinized tubes and centrifuged at 6000 rpm for 25 min. The supernatant was transferred into a microtube and frozen at -4°C until analysis time.

■ Assay validation

Partial validation experiments were carried out according to FDA recommendations [32]. The calibration, linearity, LOD, LLOQ, ULOQ intra- and inter-day precisions, accuracy, recovery, selectivity, stability (room temperature and freeze–thaw) and robustness were tested. The mean of three calibration curves (produced on three different days) was used for the validation studies.

Results & discussion

■ Optimization of spectrofluorimetry conditions

Effect of system temperature on fluorescence intensity

In spectrofluorimetry, reduction in fluorescence intensity with increasing temperature is a common phenomenon and is attributed to an increase in the number of collisions between molecules that have a quenching effect. Effects of various temperatures in the range of 0 to 40°C on fluorescence intensity of carvedilol after extraction from plasma samples were investigated. 25°C was chosen as the optimum temperature for later experiments because although by increasing the temperature from 0 to 40°C fluorescence

intensity was slightly decreased, repeatability was better at 25°C than at lower temperatures, and also because of the availability of temperature control options in some instruments.

■ Excitation & emission wavelength

The excitation and emission spectra of carvedilol were recorded at 25°C and at the excitation/emission slits of 5 nm with the scan rate of 8000 nm min^{-1} where the maximum excitation and emission wavelengths of carvedilol were at 286 and 344 nm, respectively.

■ Optimization of DLLME-SFO conditions

In order to obtain the most effective extraction and the optimum DLLME-SFO condition for the determination of carvedilol in human plasma, it is important to investigate the parameters affecting extraction, including the type and volume of extraction and disperser solvents, pH, salt addition and sample volume. For this purpose, while one parameter is changed all the other parameters are kept constant. The relative fluorescence intensity was used to evaluate the influence of the parameters under investigation on the extraction rate of carvedilol using the proposed DLLME-SFO method.

■ Selection of extraction solvent

The selection of an appropriate extraction solvent is important in the DLLME-SFO procedure. It must have low toxicity, lower density than that of water, low aqueous solubility while being soluble in disperser solvents and high boiling point (low vapor pressure) in order to reduce solvent losses during extraction. In addition, it should have a melting point near or below room temperature (in the range of 10 to 30°C). Accordingly, in order to select the best extraction solvent to attain optimized DLLME-SFO method, 1-undecanol and 1-dodecanol were selected from available solvents. By using of 100 µl of these solvents and 1.0 ml of acetonitrile (as disperser solvent), the effect of extraction solvent was investigated on extraction of carvedilol (Figure 2). There was no significant difference between extraction power of the solvents; however, 1-undecanol was solidified faster and collected easily, therefore it was selected as the extraction solvent for further experiments.

■ Selection of disperser solvent

Dissolution of disperser in both extraction solvent and aqueous sample solution is the most

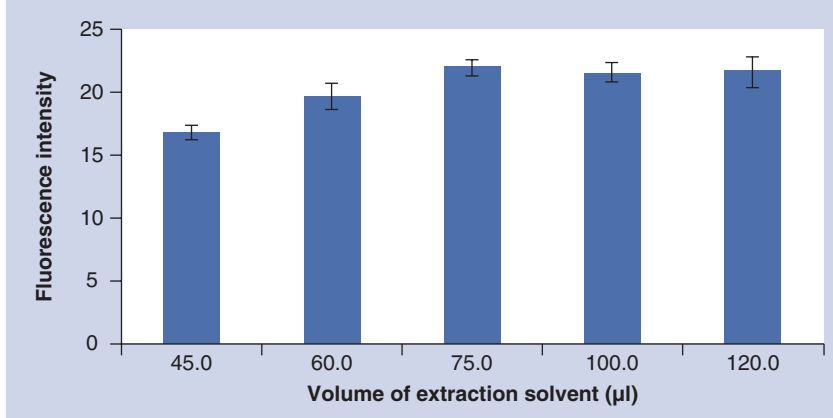

Figure 4. Effect of the extraction solvent volume on the extraction of carvedilol. Experimental conditions are the same as in Figure 3, and the optimized disperser solvent is acetonitrile.

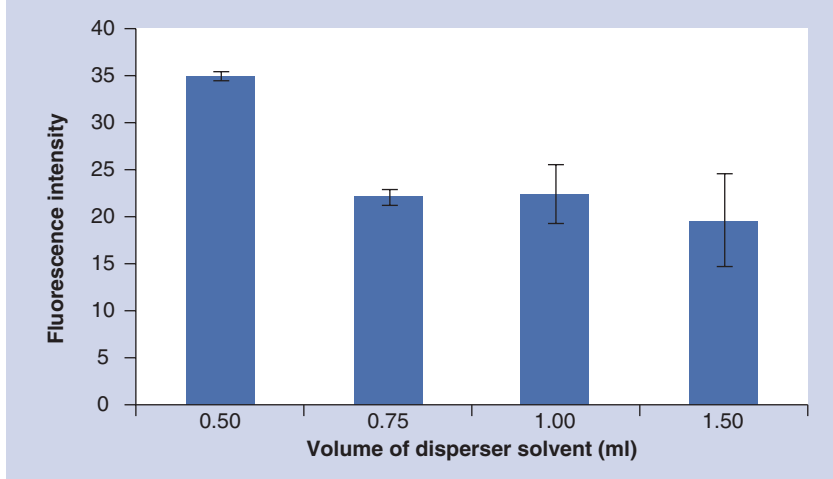

Figure 5. Effect of the disperser solvent volume on the extraction of carvedilol. Experimental conditions are the same as in Figure 4, and the optimized volume of extraction solvent is 75.0 µl.

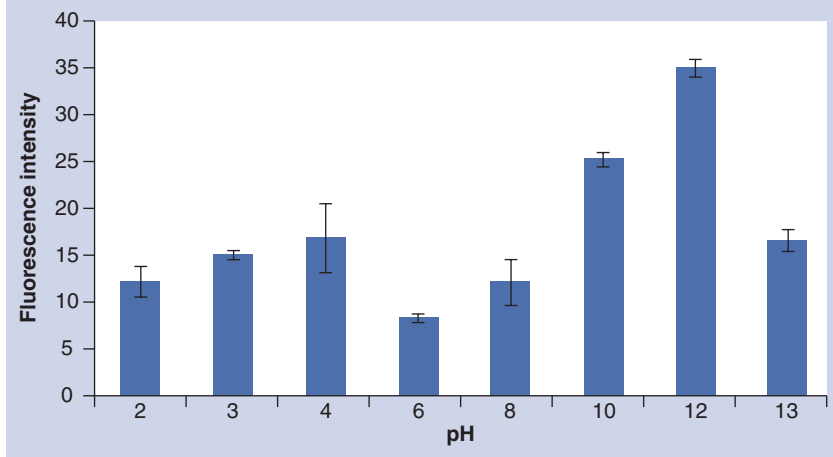

Figure 6. Effect of the sample solution pH on the extraction of carvedilol. Experimental conditions are the same as in Figure 5, and the optimized volume of disperser solvent is 0.5 ml.

important point in the choice of disperser solvent. The disperser solvent helps to produce very fine droplets of extraction solvent and disperse them throughout the aqueous sample solution, and in this way infinitely increases the contact surface with the aqueous sample solution. Acetonitrile, methanol, tetrahydrofuran (THF) and acetone were tested in this work. Extraction of carvedilol was carried out by using of 1 ml of each solvent with 100 µl 1-undecanol (as extraction solvent) The results are shown in Figure 3, and according to the results acetonitrile was selected as the disperser solvent.

Selection of extraction solvent volume

To investigate the effect of extraction solvent volume on the fluorescence signals of extracted carvedilol, micro drop volumes were investigated in the range of 45.0 to 120.0 µl, and other experimental conditions were kept unchanged. The fluorescence intensity of carvedilol increased with increasing volume of 1-undecanol from 45.0 to 75.0 µl and afterwards almost reduced and remained constant (Figure 4). An increase in the micro drop volume raises the interfacial area following the analytical signals. But further increases in the micro drop volume lead to the analytical signals almost remaining constant. With most increases of the extraction solvent volume, the total mass of the analyte in the extraction solvent is constant, and also, due to diluting of the extraction solvent to constant volume in the analysis step, therefore the signals were constant. Hence, the 75.0 µl was chosen as the optimum extraction solvent volume for later experiments. It should be noted that with lower volumes of extraction solvent, micro drops on the surface of aqueous sample solution were not formed and, thus, the method was not applicable.

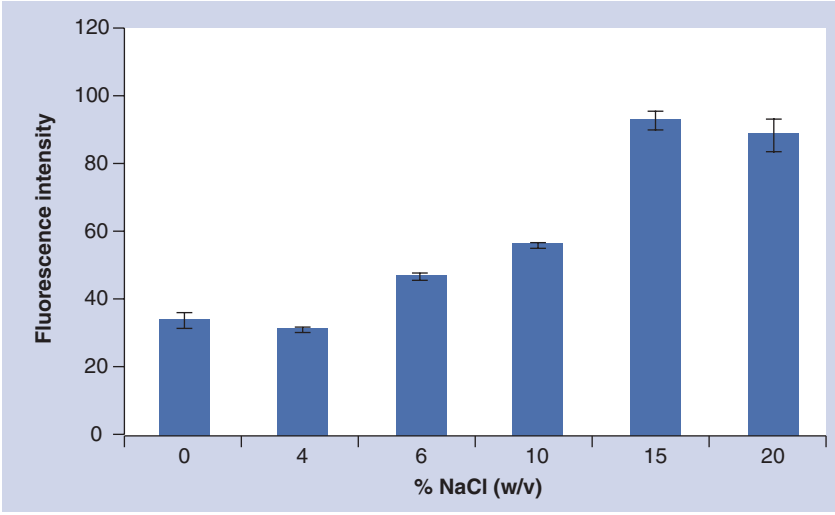

Figure 7. Effect of salt addition on the extraction of carvedilol.
Experimental conditions are the same as in Figure 6, and the optimized pH of sample solution is 12.

Selection of disperser solvent volume

The disperser solvent expands the interfacial area of the extraction solvent with the aqueous sample solution infinitely, which increases extraction rate. Therefore, the volume of disperser solvent is one factor that should be optimized. In lower volumes, the formation possibility of fine droplets of extraction solvent and the resulting cloudy state does not exist well and, thus, extraction efficiency is reduced. On the other hand, at higher volumes of disperser solvent, the solubility of analytes in aqueous sample solutions is increased because the solubility of disperser solvent in aqueous phase is often more than in the organic phase and this reduces the polarity of the aqueous phase and, thus, analyte distribution comes down to the extraction solvent and extraction efficiency is reduced. To investigate the effect of disperser solvent volume on extraction of carvedilol, various volumes of acetonitrile (0.50, 0.75, 1.00 and 1.50 ml) with 75.0 µl 1-undecanol (as extraction solvent) were assayed. By careful examination of the results shown in Figure 5, 0.50 ml acetonitrile (as disperser solvent) was chosen as the optimum volume for further studies.

Optimization of pH of aqueous sample solution

Effect of pH on the extraction of carvedilol was investigated in the range of 2.00 to 13.00 using hydrochloric acid 1 M and sodium hydroxide 1 M solutions. As can be seen in Figure 6, at pH 12.00, fluorescence intensity has reached the maximum value. Therefore pH 12.00 was chosen as the optimum pH for later studies. Concerning the pKa of carvedilol (pKa = 7.97) [34], it is not ionized at pH 8–12 and extraction should not be pH dependent. The observed variations could be justified by addition of NaOH and consequent increases in the ionic strength of the solution, which is also confirmed by the findings in the next section. This is also confirmed by logD value (2.49) of carvedilol [101].

Effect of salt addition

The effect of salt addition on the extraction of carvedilol in the range of 0 to 20.0% w/v of NaCl from spiked plasma samples was investigated, and the other parameters were kept unchanged. As

can be seen in FIGURE 7, with increasing NaCl concentration from 0 to 15.0% w/v, fluorescence intensity and, thus, extraction of carvedilol was increased. A possible reason for this observation is that water molecules form hydration spheres around the salt ions result in a reduction in the concentration of water available to dissolve analyte molecules, and thus this would drive additional analytes into the extraction solvent [35]. In other words, increasing the ionic strength of the aqueous sample solution reduces the solubility of the analyte and extraction solvent in an aqueous sample solution. Also, at higher NaCl concentrations, and especially in 15.0% w/v, micro drop formation was very good and solidified micro drops were easily collected. Based on these results, 15.0% w/v of NaCl was chosen for later studies.

Effect of sample volume

The effect of sample volume on microextraction procedure, taking into account constant dilution for all plasma samples, was studied. 1 ml of plasma after spiking and protein precipitation was used in all studied volumes. Volumes of sample solutions were changed from 2.5 to 10.0 ml in glass test tubes. Results are shown in FIGURE 8 in which the highest analytical response was achieved at the sample volume of 10.0 ml. Therefore, 10.0 ml was chosen as the optimum sample volume. Effects of speed and time of centrifugation on the analytical signals were investigated (data not shown here) and 6000 rpm and 5 min were chosen for further studies.

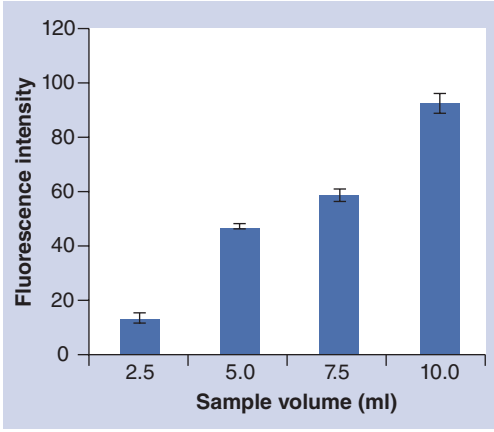

Figure 8. Effect of the sample volume on the extraction of carvedilol. Experimental conditions are the same as in FIGURE 7, the optimized salt amount is 15.0% w/v and speed and time of centrifuging is 6000 rpm and 5 min, respectively.

Calibration curve & linearity

After optimization of all parameters, the calibration curve was plotted on three different days at seven increasing levels and the mean of three curves was used for validation studies. It can be seen that there is a good linear relationship between the fluorescence intensity and the concentration of carvedilol in the range of 0.040 to 0.300 µg ml^{-1}. Typical equation for mean calibration curve was: y = 0.397x + 7.131, and the correlation coefficients were >0.996 for all three calibrations. The LOD for the method in human plasma were obtained based on $C_{LOD} = 3S_b/m$ (S_b: standard derivation of blank, m: slope of calibration curve). The details of mean calibration curve and amounts of LOD, LLOQ and ULOQ are shown in TABLE 2. Comparing these findings with those listed in TABLE 1 reveals that the previous methods provided better validation data. However, the main advantage of the proposed method is its simplicity and low cost, which makes it a suitable analytical method for routine applications in biomedical analysis laboratories.

Precision & accuracy

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume

Table 2. Validation data of the proposed method for quantification of carvedilol in human plasma.

Parameters	Level
Linear range (µg ml^{-1})	0.040–0.300
Slope (standard error of mean)	0.397 (0.016)
Intercept (standard error of mean)	7.131 (2.800)
Correlation coefficient	0.996
Number of data points	7
LOD (ng ml^{-1})	18
LLOQ (ng ml^{-1})	40
ULOQ (ng ml^{-1})	300

Key Term

Biological fluids: Liquids originating from inside the bodies of living people. They include fluids that are excreted or secreted from the body.

Table 3. Assay precision and accuracy of QC samples if assuming the optimal extraction and analysis conditions.

Nominal concentration (ng ml^{-1}; n = 5)	Intra-assay precision (RSD%; n = 5)	Inter-assay precision (RSD%; n = 5)	Accuracy (RE%)
50	8.7	6.9	-0.7
150	7.7	9.9	2.5
250	8.5	2.1	1.4

RE: Relative error.

of biological matrix. The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analyses of samples containing known amounts of the analyte. Precision and accuracy should be measured using a minimum of five determinations per concentration. The precision and accuracy determined at each concentration level should not exceed 15% of the relative standard deviation (RSD%), except for the LLOQ where it should not exceed 20% of the RSD [32]. Accuracy and precision of the assay should be determined for both intra- and inter-runs. They are determined by analyzing quality control (QC) samples at a minimum of three concentrations (low, mid and high), representing the entire range of the calibration curve [36]. Inter- and intra-assay precisions along with accuracy for QC samples are listed in Table 3. All RSD% were below 10% for spiked plasma samples. The results obtained for these validation experiments showed that the developed method is both accurate and precise.

Recovery

Plasma samples were spiked with three QC concentration levels (low, mid and high) of carvedilol. Protein precipitation and DLLME-SFO procedures were carried out, and the mean recoveries for carvedilol obtained and summarized in Table 4. The mean recovery for carvedilol was 100.3%.

Stability

Drug stability in a **biological fluid** is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system [32]. The stability results are summarized in Table 5 where the highest deviation was obtained for 50 ng ml^{-1} of carvedilol at room temperature (relative error % = 14.7%), which is acceptable (<20%) for biological samples according to FDA recommendations [32].

Robustness

Robustness evaluates the ability of an analytical method against small changes in practical effective parameters that may be increased or

Table 4. Recoveries for extraction and analysis of carvedilol in spiked plasma samples with DLLME-SFO-spectrofluorimetry.

Nominal concentration (ng ml^{-1}; n = 5)	Found concentration (ng ml^{-1}; ±σ; n = 5)	Recovery (%)
50	50 (±4)	99.3
150	154 (±15)	102.5
250	254 (±5)	101.4

Table 5. Stability data for carvedilol in human plasma samples.

Nominal concentration (ng ml^{-1}; n = 3)	Room temperature stability			Freeze–thaw stability		
	Mean (±σ) found concentration (ng ml^{-1})	Accuracy (RE%)	Recovery (%)	Mean (±σ) found concentration (ng ml^{-1})	Accuracy (RE%)	Recovery (%)
50	57 (±10)	14.7	114.7	55 (±20)	9.4	109.4
150	166 (±5)	10.9	110.9	149 (±23)	-0.9	99.1
250	281 (±15)	12.5	112.5	275 (±3)	10.1	110.1

RE: Relative error.

Table 6. Results of the evaluation of method robustness in three different levels.

Level	Nominal concentration (ng ml^{-1}; n = 3)	Mean found concentration (ng ml^{-1}; n = 3)	Accuracy (RE%)	Recovery (%)
1	150	140	-6.4	93.6
2	150	140	-6.9	93.1
3	150	146	-3.0	97.3

1: pH = 12.20, 15.5% (w/v) NaCl, speed and time of centrifugation: 7000 rpm for 6 min.
2: pH = 11.80, 14.5% (w/v) NaCl, speed and time of centrifugation: 5000 rpm for 4 min.
3: pH = 12.00, 15.0% (w/v) NaCl, speed and time of centrifugation: 6000 rpm for 5 min.
RE: Relative error.

decreased during the operations. The results are shown in Table 6. The results showed negligible differences in the obtained data and demonstrated that the developed method is a robust method.

Selectivity

The selectivity of the developed method was evaluated using some other co-administered cardiovascular drugs under the optimum conditions, and the changes in fluorescence intensity (ΔF%) were calculated. Results are shown in Table 7 in which most coexisting drugs show no interference.

Application to real samples

In order to evaluate the feasibility and applicability of the developed method in the analysis of real plasma samples for the determination of carvedilol, 22 different plasma samples from patients were analyzed by the proposed method and the results, along with some details of the patients, are summarized in Table 8. It should be added that the reported concentrations are total concentration of carvedilol since the drugs are released in the process of protein precipitation by organic solvents [37].

Conclusion

A new combined methodology of DLLME-SFO and spectrofluorimetric determination was applied to the extraction and analysis of carvedilol in human plasma samples. In this study using low-density and low-melting-point organic solvent (1-undecanol) as the extraction solvent meant that the extraction micro drop could be easily collected by solidifying, which facilitated the phase transferring. Also, the use of low-toxicity extraction solvent instead of solvents with high toxicity in DLLME is environmentally friendly. The proposed DLLME-SFO method has an excellent clean-up effect and is able to eliminate the plasma matrix effect. In other words, this microextraction method is one of the most appropriate methods for eliminating the effects of complex matrices, such as plasma, while it is also very low cost, easy and fast. The DLLME-SFO-spectrofluorimetric method permits the fast, accurate and reliable determination of carvedilol in human plasma with low operation cost and simplicity of instrumentation. It is obvious that in drug bioequivalence studies in which the volunteer were given a single dose of a drug and the changes in drug concentrations is plotted versus time, the proposed method is not suitable for low concentrations. The developed method has good precision, selectivity, stability and robustness and can be applied successfully as

Table 7. Evaluation of effect of other drugs on extraction of carvedilol (50 ng ml^{-1}).

Drug	Concentration of interfering drug (μg ml^{-1})	ΔF%
Aspirin	50	-0.1
Atorvastatin	50	-13.4
Atorvastatin	5	-9.5
Captopril	50	-2.8
Clopidrogel	50	-13.0
Clopidrogel	5	0.6
Digoxin	50	1.7
Enalapril	50	9.8
Furosemide	50	-2.1
Hydrochlorothiyazide	50	4.0
Losartan	25	-0.5
Lovastatin	50	-4.5
Nitroglycerin	50	-15.8
Nitroglycerin	5	-6.2
Omeprazole	50	-19.2
Omeprazole	5	3.6
Oxazepam	50	-1.1
Pantoprazole	50	-1.1
Spironolactone	50	4.4
Valsartan	50	-1.7
Warfarin	50	-27.9
Warfarin	5	2.4

Table 8. Some details of the real samples analyzed by the proposed method and their carvedilol concentration.

No.	Gender	Age	Carvedilol daily dosage (mg)	Duration of dairy intake	Co-administered drugs	Intake time	Sampling time	Concentration (ng ml^{-1})
1	Male	45	6.25 b.i.d.	3 months	Atorvastatin, lozartan, digoxin, clopidogrel and aspirin	8 am	2 pm	155
2	Female	62	25 b.i.d.	2 months	Clopidogrel, aspirin, folic acid, oxazepam, furosemide and atorvastatin	9:30 am	11:30 am	164
3	Female	68	6.25 b.i.d.	3 years	Captopril, alprazolam and furosemide	11 am	11:10 am	44
4	Female	65	3.125	5 months	Omeprazole, spironolactone, clopidogrel, atorvastatin, captopril and nitroglycerin	10:30 am	11:30 am	96
5	Female	60	6.25 b.i.d.	1 month	Valsartan	7 am	11 am	78
6	Male	54	6.25 b.i.d.	3 months	Clopidogrel, lozartan, aspirin and digoxin	10 am	2:30 am	95
7	Male	73	12.5 b.i.d.	4 days	Spironolactone, digoxin, lozartan, aspirin and furosemide	9 am	11 am	156
8	Female	87	3.125 b.i.d.	1 year	Aspirin, furosemide, enalapril and amlodipine	9 am	11 am	149
9	Male	73	3.125 b.i.d.	3 months	Omeprazole, spironolactone, aspirin, furosemide, digoxin and captopril	9 am	10 am after 2 days	216
10	Male	71	12.5 b.i.d.	1 month	Omeprazole, furosemide, clopidogrel, oxazepam, atorvastatin, captopril and nitroglycerin	9 am	2:15 pm	223
11	Female	82	6.25 b.i.d.	1 week	Omeprazole, furosemide, clopidogrel, oxazepam, atorvastatin, captopril, nitroglycerin and aspirin	10 am	2 pm after 1 day	66
12	Male	65	6.25 b.i.d.	4 days	Omeprazole, furosemide, aspirin, spironolactone, atorvastatin, vaptopril, nitroglycerin and lozartan	8 am	5 pm	48
13	Male	67	6.25 b.i.d.	4 days	Pantoprazole, aspirin, atorvastatin, captopril, phenazopyridine, omeprazole and clopidogrel	9 am	9 am	150
14	Female	63	6.25 b.i.d.	8 days	Omeprazol, lozartan and lovastatine	9 am	4:30 pm	56
15	Male	59	6.25 b.i.d.	4 years	Aspirin, captopril, nitroglycerin and spironolacton	8:30 am	11:10 am	89
16	Male	49	3.125 b.i.d.	6 years	Furosemide, digoxin, aspirin and triamtern H	8 am	3:45 pm	40
17	Female	68	3.125 b.i.d.	20 days	Furosemide, aspirin and nitroglycerin	8 am	5 pm	117
18	Male	64	6.25 b.i.d.	Many years	Aspirin, atorvastatin and captopril	8 am	6 pm	92
19	Male	85	6.25 b.i.d.	Many months	Lozartan, aspirin, digoxin and hydrochlorothiazide	7 am	10:30 am	69
20	Female	49	3.125 b.i.d.	13 days	Furosemide, spironolactone, glibenclamide metformin, atorvastatine, enalapril, clopidogrel amd omeprazole	8 am	12 am	189
21	Male	23	6.25 b.i.d.	2 years	Digoxin, warfarin, atorvastatin, captopril and furosemide	8 am	10:30 am	57
22	Male	56	6.25 b.i.d.	2 years	Furosemide, aspirin, atrovastatin and nitroglycerin	7:30 am	11:30 am	91

b.i.d.: Twice-daily dosing.

a routine analytical method in therapeutic drug monitoring.

Future perspective

The use of the DLLME-SFO-spectrofluorimetry technique for the analysis of drugs in different biological samples and in bioanalytical laboratories will provide quick analysis of trace amounts of drugs in these matrices without needing expensive equipment and toxic solvents.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Aim

- A dispersive liquid–liquid microextraction based on solidification of floating organic droplet spectrofluorimetry method was developed for the extraction and determination of carvedilol in human plasma samples. The effect of various parameters was investigated for the optimization.

Procedure

- Take 700 µl of plasma.
- Add 700 µl acetone.
- Take 1 ml of supernatant after centrifugation.
- Add 15.0% (w/v) NaCl, adjust pH to 12.00 and dilute with water up to 10 ml in a volumetric flask.
- Add dispersive solvent and extraction solvent to the above solution in a glass test tube and centrifugate it.
- Take the solidified extracted phase from the top of the tube after cooling and then melt it.
- Mix with methanol up to 1 ml in a microtube and transfer to spectrofluorimetry system.

Results & discussion

- US FDA guidelines were used for validation of the developed method.
- This developed method is applicable to the analysis of carvedilol in plasma samples.
- The DLLME-SFO method is applicable to other biological samples.
- The developed method was applied to the analysis of some real plasma samples obtained from various cardiac patients.

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