Hollow Fiber Based Liquid Phase Microextraction with High Performance Liquid Chromatography for the Determination of Trace Carvedilol (β-Blocker) in Biological Fluids

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A hollow-fiber liquid-phase microextraction (HF-LPME), followed by high-performance liquid chromatography–ultraviolet (HPLC–UV) method for the trace determination of carvedilol (β-blocker) in biological fluids, has been described. The separation was achieved using Inertsil ODS-3 C18 (250 mm × 4.6 mm, 3 μm) column with a mobile phase composition of 10 mM phosphate buffer (pH 4.0)–acetonitrile (50:50, v/v) at a flow rate of 1.0 mL/min, under isocratic elution. Several parameters (i.e., type of organic solvent, donor phase pH, concentration of acceptor phase (AP), stirring rate, extraction time, and salt addition) that affect the extraction efficiency were investigated. The optimum HF-LPME conditions were as follows: dihexyl ether as an organic solvent; donor phase pH, 10.7; 0.1 M HCl (AP); 1100-rpm stirring rate; 60-min extraction time; and no salt addition. These parameters have been confirmed using design of experiments. Under these conditions, an enrichment factor of 273-fold was achieved. Good linearity and correlation coefficient were obtained over the range 5–1000 ng/mL (r² = 0.9994). Limits of detection and quantitation were 1.2 and 3.7 ng/mL, respectively. The relative standard deviation at 3 different concentration levels (5, 500, and 1000 ng/mL) were less than 13.2%. Recoveries for spiked urine and plasma were in the range 80.7–114%. The proposed method is simple, sensitive, and suitable for the determination of carvedilol in biological fluids.

Keywords: hollow fiber-liquid phase microextraction, high-performance liquid chromatography, carvedilol, biological fluids, design of experiments

1. Introduction

Carvedilol (CVD), (±)-1-(9H-carbazol-4-yl-oxo)-3-[[2-(2-methoxyphenoxy)ethyl]amino]propan-2-ol (Figure 1), is a highly lipophilic drug (log P, 4.19) with pKa values of 8.74 and 14.03 [1]. CVD is universally used as nonselective α and β-blocking activity to treat cardiovascular diseases such as hypertension, mild-to-severe congestive heart failure, and angina pectoris [2, 3]. Furthermore, it exhibits antioxidant activity which can help to protect organs such as heart and kidney from damage [4]. The bioavailability of its oral intake (50-mg dose) was found to be 24% [5]. CVD is extensively metabolized in the human body mostly by liver via oxidative and conjugative pathways [2, 6]. Less than 2% of the dose will be excreted in urine as unchanged drug [6]. The maximum plasma concentration of CVD is 72.2 ng/mL [7]. In plasma, CVD is mainly bound to proteins (>98%) [8].

Several analytical methods have been reported for the determination of CVD in biological fluids and pharmaceutical formulations [3, 8–26]. Chromatographic methods, such as high-performance liquid chromatography (HPLC) with ultraviolet (UV) [3, 8–11] or fluorescence (FL) [12–16] detection, are the most common technique for the analysis of CVD. Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) have also been reported [2, 17–19]. The matrix effects, long analysis time, low sensitivity, and moderate specificity are the main disadvantages of the LC methods [9]. Gas chromatography with mass spectrometry (GC–MS) was used as alternative chromatographic technique for the determination of CVD in biological fluids [20, 21]. The need for derivatization is the main disadvantage of GC methods, which is due to the poor volatility of CVD. Non-chromatographic methods,

Figure 1. Chemical structure of carvedilol (CVD)

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such as fluorimetry and flow injection analysis, have been also reported [22–28]. Although these methods provide short analysis time, severe interferences, poor selectivity, and sensitivity are the main drawbacks of these techniques.

In order to overcome the limitations in the analysis, sample preparation is considered mandatory, especially for biological fluid samples. Liquid–liquid extraction (LLE) [6, 7, 16, 18–21, 29–31] and protein precipitation (PP) [13, 14, 17, 22] are the most widely used extraction techniques. The consumption of large amounts of toxic and expensive organic solvents (e.g., dichloromethane, diethyl ether, methyl t-butyl ether, and hexane), time-consuming, and interferences are the main limitations of these techniques. Solid-phase extraction (SPE) and supported liquid extraction (SLE) have also been reported for the extraction of CVD in biological fluids [16, 29]. These techniques have the potential to provide clean extracts and require considerable amounts of organic solvents but are time-consuming due to the multi-extraction steps. Furthermore, low enrichment of the analytes is the norm.

Microextraction techniques for drug analysis have gained lots of attention because of their promise in providing high enrichment of the analytes using minimum volume of organic solvents. To date, dispersive liquid–liquid microextraction (DLLME) [9, 27] and stir bar sorptive extraction (SBSE) [3] methods are the only microextraction techniques used for the extraction of CVD in biological fluids. Although these techniques are promising, the multi-extraction steps, long evaporation process, carryover of analytes, and the use of toxic solvents are still involved [3, 9, 27]. Hollow-fiber liquid-phase microextraction (HF-LPME) exhibits many advantages compared to the other microextraction techniques in terms of simplicity and minimum consumption of organic solvents [32]. It is also cheap as the fiber can be disposed once used, thus minimizing carry over problems. The technique provides clean extracts and high enrichments using small injection volume (1–5 μL).

In the current study, for the first time, a three phase HF-LPME with HPLC–UV method has been developed for the determination of CVD. Various parameters that influence the extraction efficiency were investigated and optimized. The method was validated for the determination of CVD in biological fluid samples (urine and plasma).

2. Materials and Methods

2.1. Chemical and Reagents. Carvedilol standard was kindly donated by Hikma Pharmaceuticals (Amman, Jordan). Methanol (HPLC grade, ≥99.96%), 2-propanol (99.5%) and hydrochloric acid (37%, w/w) were purchased from Merck (Darmstadt, Germany). Acetonitrile (HPLC grade, 99.99%) was purchased from Fisher Scientific (Milwaukee, USA). Diisopropyl ether (97.0%), n-heptane (99.0%), n-hexadecane (99.0%), sodium phosphate monobasic monohydrate, sodium phosphate tribasic dodecahydrate (5.1 mg) in water (1.0 L).

2.2. Instrumentation and Chromatographic Conditions. Separation was conducted using a Hitachi LC-6200 intelligent pump (Tokyo, Japan) equipped with a Hitachi L-4250 UV–VIS detector (Tokyo, Japan). Detection wavelength was set at 285 nm. Samples were introduced to the system via a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 5-μL loop. A PowerChrom data acquisition was obtained from dEQ (Denistone East, Australia) and performed with PowerChrom software v2.6.11. The separation was achieved using an Inertsil ODS–3 C18 (250 mm × 4.6 mm, 3 μm) analytical column purchased from GL Sciences (Tokyo, Japan) with a mobile phase composition of 10 mM sodium phosphate buffer (pH 4.0)–acetonitrile (50:50, v/v) at 1.0 mL/min, under isocratic elution. The mobile phase was filtered using a nylon membrane filter (0.22 μm) from Agilent Technologies (Waldbrommn, Germany) and degassed for 15 min before use.

2.3. Preparation of Standard Solutions. Stock solution (2000 μg/mL) was prepared in methanol and stored at 4 °C until use. Working standard solutions were prepared daily by suitable dilution of the stock solution in water with pH adjusted to 10.7 using NaOH (0.1 M). Working standard solutions were used as the donor phase (DP) in the microextraction procedure.

2.4. Minimization of Protein Binding in Plasma and Urine. In order to eliminate the high protein binding between plasma and CVD, pretreatment steps adopted form the work of Al Arfaj et al. with some modification were used [22]. Firstly, drug-free plasma (1 mL) was spiked with CVD at the desired concentration. Methanol (5 mL) was added and vortex-mixed for 2 min. The mixture was centrifuged for 5 min at 2600 rpm. The supernatant was collected and evaporated to dryness under a gentle nitrogen stream at 50 °C. The dry residue was dissolved in methanol (2 mL), and the mixture was then centrifuged for 5 min at 2600 rpm. The supernatant was diluted with water to 10 mL with pH adjusted to 10.7 using NaOH (0.1 M). The urine sample (5 mL) was spiked with CVD at the desired concentration and diluted with water to 10 mL with pH adjusted to 10.7. Urine and plasma solutions (DP) were then subjected to the HF-LPME procedure.

2.5. HF-LPME Procedure. The HF segments (4 cm each) were sonicated in acetone for 15 min and left to dry overnight before use. The working standard or sample solution (10 mL) was transferred to a sample vial (12 mL) containing a magnetic stirring bar (15 mm × 5 mm). HCl (10 μL, 0.1 M) as the acceptor phase (AP) was withdrawn using a 25-μL Hamilton microsyringe (model 702SNR). The microsyringe needle and needle tip (21G × 38 mm) were inserted into the HF segment, and it was bent to U-shape. The HF was immersed in diisopropyl ether (organic phase) for 10 s to impregnate its pores wall. The HF was then soaked in water for 5 s to remove the extra organic solvent and immediately immersed into the sample solution (DP). The AP was injected into the lumen of the HF, and the extraction was carried out for 60 min at 1100 rpm stirring rate. At the end of the extraction, a 5-μL extract was withdrawn by the microsyringe and injected into the HPLC unit. The HF was disposed after single use. A multi-stirrer (model GLHPS-G) purchased from Global Lab (South Korea) was used.

2.6. Design of Experiments. In order to confirm the optimum conditions obtained by the traditional experimental
optimization, a two-level fractional factorial experimental design (full fractional) was used to evaluate the preliminary significance of the factors and their interactions. The factors investigated were the sample pH, HCl concentration, stirring speed, and extraction time. All factors were evaluated at low (as $-1$) and high (as $+1$) levels in 3 replicates. The number of experiments ($n$) was 16, which is given by the expression: $n = 2^k$, where $k$ is the number of factors.

The most significant factors indicated by the Pareto chart were optimized using a Box–Behnken design (response surface methodology) based on 3-level factorial design. The number of experiments ($n$) was 20, which is calculated by the equation: $n = 2^k(k - 1) + C$, where $k$ is the number of factors, and $C$ is the replicate number of center points. All factors was studied at 3 different levels (low, medium, and high, coded as $-1$, 0, and $+1$, respectively). Experimental design levels are summarized in Table 1.

### Table 1. Factors and levels investigated using Box-Behnken experimental design

| Factor          | Coded | $-1$ | 0  | $+1$ |
|-----------------|-------|------|----|------|
| Sample pH       | $x_1$ | 9.7  | 10.7 | 11.7 |
| Stirring speed (rpm) | $x_2$ | 600  | 900  | 1200 |
| Extraction time (min) | $x_3$ | 20   | 47.5 | 75   |

3. Results and Discussion

To separate CVD, initial conditions (i.e., 15 mM sodium dihydrogen phosphate buffer (pH 4.0)–acetonitrile–2-propanol (70:27.5:2.5, v/v) as the mobile phase, a flow rate of 1.0 mL/min, and a Hypersil ODS–C$_{18}$ column (250 x 4.6 mm, 5 μm particle size)) adopted from the work of Soltani et al. were used [9]. Detection wavelength was set at 285 nm instead of 222 nm in order to reduce interferences. Under these chromatographic conditions, long retention time (~10 min) and broad peak were observed. Alternatively, an Inertsil ODS 3–C$_{18}$ column (250 x 4.6 mm, 3 μm) was used. An improvement in the peak shape was obtained but the retention time was still long (~9 min).

To reduce the retention time and simplify the chromatographic conditions, the mobile phase was optimized. A composition of acetonitrile–water and acetonitrile–15 mM phosphate buffer (pH 4.0) was tested. The best peak shape and retention time were found when acetonitrile–15 mM phosphate buffer (pH 4.0) (50:50, v/v) was used as the mobile phase. Therefore, buffer concentration and pH was subjected for further investigations. 10 mM buffer concentration at pH 4.0 exhibited the best separation. Under these conditions, a sharp peak with retention time of about 3.9 min was achieved.

3.1. Optimization of HF-LPME Conditions. Several parameters, such as type of organic solvents, DP pH, concentration of the AP, stirring speed, extraction time, and salt addition, which affect the extraction efficiency of the HF-LPME, have been investigated.

Organic solvents used in HF-LPME should be water-immiscible and easily immobilized within the pores of the polypropylene fiber, have good affinity for the analyte, and low volatility to avoid evaporation during the extraction [33–35]. Eight organic solvents were investigated, i.e., dihexyl ether, 1-heptanol, 1-octanol, $n$-heptane, $n$-octane, $n$-decane, $n$-tridecane, and $n$-hexadecane (Figure 2(A)). Dihexyl ether gives the highest extraction efficiency. Thus, it was selected for the subsequent experiments.

The pH of the DP should be adjusted to ensure that CVD will exist in the neutral form in order to be effectively extracted to the organic phase [34,35]. As CVD has a weak basic ($-\text{MH}^-$, $pK_a = 8.74$) and very weak acidic ($-\text{OH}$, $pK_a = 14.03$) functional groups, the pH of the DP should be adjusted higher than the basic but lower than the acidic $pK_a$ values by at least 2–3 units. The effect of the DP pH (9.7–11.7) was thus studied.

![Figure 2](image-url)
The obtained results showed that the extraction efficiency increased as the pH increased from 9.7 to 10.7, where the degree of deionization is increased (Figure 2(B)). At high pH (>10.7), –OH group will be partially ionized. Thus, the solubility of CVD will increase, and extraction efficiency decrease. Therefore, pH 10.7 was selected.

Analyte in the AP should exist in the ionized form to prevent the back extraction to the organic phase [34–36]. As CVD contains weak basic and very weak acidic functional groups, the AP should be a weak acid or strong base to assure that the analyte exists in the ionized form. The use of strong base as AP was not recommended due to the detrimental effect on the chromatographic system and column. Therefore, different concentrations of HCl (0.01–0.10 M) have been investigated. It was found that the extraction efficiency increased as the concentration of HCl increased. This is due to the increase of the degree of ionization. Higher concentration of HCl was not recommended to maintain a long lifetime of the column and instrumental parts [35, 36]. Therefore, 0.10 M HCl was used for further investigations.

Agitation of the DP was reported to enhance the mass transfer of the analyte by speeding-up the thermodynamic equilibrium and allow the continuous exposure of the extraction surface to the sample solution [35–37]. The effect of stirring speeds (800–1200 rpm) on the extraction efficiency has been investigated. The extraction efficiency was found to increase from 800 to 1100 rpm and decrease thereafter (Figure 2(C)). The decrease in the extraction efficiency at a high stirring speed (>1100 rpm) is probably due to the formation of air bubbles [37]. A rate of 1100 rpm was selected for the subsequent experiments.

Extractions using HF-LPME typically requires 20 min or more [35–37]. In this work, extraction time from 20 to 75 min has been studied. The extraction efficiency was found to increase with time (Figure 2(D)). This is due to the low partitioning of CVD into the aqueous phase (Log P, 4.19), which slow the mass transfer of CVD from the organic phase to the AP. An extraction time of 60 min was sufficient to obtain good equilibrium and extraction efficiency (enrichment factor, 273-fold). Thus, 60 min extraction time was selected. Although the extraction time was relatively long, many extractions can be simultaneously performed by using multi-stirrers.

Salt is usually added to the DP to enhance the partitioning of analyte into the organic phase (salting-out effect) [35]. However, the effect of salt addition depends on the nature of the analyte. In this study, 0–20% (w/v) of NaCl has been added into the sample solution. It was observed that the extraction efficiency decreased as the percentage of NaCl increased. This is due to the change in the physical properties of the extraction film in the presence of salt with high concentrations [37]. Furthermore, the increment of the viscosity of the bulk solution reduced the diffusion rate of the analyte, thus restricting the movement of the analyte to the organic phase [34–36]. Therefore, extraction was carried out without adding salt.

The adopted extraction conditions were the following: dihexyl ether as organic solvent; pH of the DP, 10.7; AP, 0.1 M HCl; stirring rate, 1100 rpm; extraction time, 60 min; and no addition of salt. Enrichment factor (EF) as an indicator of the extraction efficiency was calculated using the formula:

\[
EF = \frac{C_{ap}}{C_d}
\]

where \(C_{ap}\) is the concentration of analyte in the AP, and \(C_d\) is the concentration of analyte in the DP. Under the optimum conditions, an EF of 273-fold has been achieved. Typical chromatogram is shown in Figure 3.

**Figure 3.** Typical chromatogram of CVD under the optimum extraction conditions: organic solvent, dihexyl ether; donor phase pH, 10.7; acceptor phase, 10 \(\mu\)L (0.1 M HCl); stirring speed, 1100 rpm; extraction time, 60 min; and no salt addition

### 3.2. Design of Experiments

In this study, design of experiments was used in order to confirm the optimum conditions that were obtained by the traditional experimental optimization. Firstly, the fractional design method was used in order to focus on the significant factors that influence the extraction efficiency (EF). The obtained results by Pareto chart from the fractional design showed that sample pH, stirring speed, and extraction time have the most significant effect on the EF; therefore, Box–Behken design was used to study these parameters by maintaining the concentration of HCl at 0.1 M as the optimum concentration of AP. A second order polynomial model that correlated the relationship between response (EF) and extraction factors was established based on the obtained experimental data. These relationships can be expressed by the following equation (Eq. 1):

\[
EF = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2
\]

where EF is the predicted response (EF); \(\beta_0\) is the intercept; \(X_1, X_2,\) and \(X_3\) are the independent factors; \(\beta_1, \beta_2,\) and \(\beta_3\) are the linear coefficients; \(\beta_{12}, \beta_{13},\) and \(\beta_{23}\) are the cross product coefficients; and \(\beta_{11}, \beta_{22},\) and \(\beta_{33}\) are the quadratic term coefficients.

The values of intercept and all of the coefficients of the second polynomial model were obtained by the multiple nonlinear regression analysis on the experimental data. The empirical relationship between the response and the tested factors was obtained using the response surface methodology. The response (EF) and the tested factors were found to be related, with a determination coefficient \((r^2)\) of 0.9754, by the following second-order polynomial equation (Eq. 2):

\[
EF = -7954 + 1449.7X_2 + 0.562X_2 + 1.10X_3 + 0.0033X_1X_2 + 0.116X_2X_3 + 0.001419X_2X_3 - 67.83X_1^2 - 0.000328X_2^2 - 0.01244X_3^2
\]
The optimum values of $X_1$, $X_2$, and $X_3$ corresponding to the maximum EF were obtained by solving the previous equation (Eq. 2). For the same reason that has been discussed in the traditional experimental optimization, the maximum value of the extraction time ($X_3$) has been held at 60 min. The optimum values of the tested parameters were $X_1 = 10.77$ min, $X_2 = 1042.24$ min, and $X_3 = 60$ min with EF equal to 242.9 (Figure 4). Equation 2 was also used to predict the EF for the optimum parameters obtained by traditional experimental optimization ($X_1 = 10.7$, $X_2 = 1100$ rpm, and $X_3 = 60$ min), and it was found to be 241.5, which is very close to the EF (242.9) obtained by solving the second-order polynomial equation. Thus, both optimum parameters can be used in this study.

3.3. Method Validation. Linearity was studied by performing the extraction for 6 concentration levels over the range 5–1000 ng/mL, and each concentration was carried out thrice. Calibration curve has been constructed by plotting the peak area (y-axis) as a function of the analyte concentration (x-axis). Calibration curve was well correlated over the studied range with a regression equation of $y = 1.374x - 6.701$ ($r^2 = 0.9994$). Repeatability study was performed for 6 replicate extractions at 3 different concentration levels (5, 500, and 1000 ng/mL), and the results were expressed as relative standard

| C<sub>added</sub> (ng/mL) | C<sub>found</sub> (ng/mL) | Recovery (%) ± SD |
|--------------------------|--------------------------|-------------------|
| Plasma                   |                          |                   |
| 5.0                      | 5.2                      | 104 ± 11          |
| 500                      | 500                      | 100 ± 11          |
| 1000                     | 807                      | 80.7 ± 12         |
| Urine                    |                          |                   |
| 5.0                      | 5.7                      | 114 ± 17          |
| 500                      | 465                      | 93.0 ± 3.8        |
| 1000                     | 873                      | 87.3 ± 5.3        |

Table 2. Recoveries obtained by spiking the biological fluid samples with CVD standard and subjected to the proposed HF-LPME–HPLC–UV method ($n = 3$)
deviations (RSDs). The obtained RSDs of the peak areas were 13.2, 8.18, and 8.23%, respectively. Limits of detection (LOD) and quantitation (LOQ) were calculated at a signal-to-noise (S/N) ratio of 3 and 10, respectively. The LOD and LOQ of the proposed method were 1.2 and 3.7 ng/mL, respectively.

3.4. Recovery Study. In order to prove the applicability of the proposed method, recovery study was performed by preparing three replicate extractions of spiked plasma and urine at three different concentration levels (5, 500, and 1000 ng/mL). Good recoveries (80.7–114%) were observed for plasma and urine samples as shown in Tables 1 and 2. Clean chromatograms of the plasma and urine extracts can be clearly seen (Figures 4 and 5). No interferences were observed. This proved the effectiveness and the high selectivity of the proposed HF-LPME method.

3.5. Comparison with Other Analytical Methods. Analytical characteristics of the proposed method over the other reported were compared (Tables 2 and 3). A wide linear range (5–1000 ng/mL) of the reported method compared to the previously reported methods [3, 12, 27] can be seen. The LOQ (3.7 ng/mL) was lower than the reported methods using traditional extraction technique, such as LLE with GC–MS [20], PP with flow injection–chemiluminescence, [22] and a recent extraction technique, i.e., DLLME with HPLC–UV or spectrofluorometry [9, 27]. However, it was higher than the previously reported method using SPE with HPLC–FL [16], SBSE with HPLC–UV [3], and LLE with LC–MS/MS [18, 19]. Generally, the method showed good sensitivity compared to the reported methods using more sensitive detectors such as fluorescence, MS, or ultraviolet at a lower wavelength.

Recoveries obtained were acceptable (80–120%), similar to the previous reported methods. The recovery for urine was lower than the reported method using DLLME with HPLC–UV [9]. The best recovery for plasma samples can be clearly seen in the reported DLLME with spectrofluorometry methods (98.2–102%), and other reported recoveries were comparable with the recoveries in this study. The green approach of the presented method can be clearly enjoyed in the analysis compared to all the previously reported methods. Furthermore, the proposed method overcomes the carry over problems in the SBSE as the fiber is disposed after each extraction [3].

4. Conclusions
A HF-LPME with HPLC–UV method for the determination of CVD has been for the first time reported. The method was optimized and validated for the determination of CVD in biological fluids. The high enrichment factor (273-fold) obtained leads to low limit of quantitation using a small injection volume (5 μL). The green approaches of the proposed method can be realized by the minimization of chemical consumption and waste generation compared to the previously reported method. Also, the application of design of experiments was a possible, efficient, rapid, and economical way of optimization strategy in order to achieve more green approaches. This method is simple and cheap and can be used with other sensitive detectors (e.g., chemiluminescence and fluorescence). Furthermore, the developed method can be extended for the determination of CVD and its metabolites after oral administration.

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