Determination of Carvedilol Enantiomers in Pharmaceutical Dosages by SBSE–HPLC Based on Diastereomer Formation

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A sensitive, selective and simple method for the simultaneous determination of carvedilol enantiomers in aqueous solution has been developed using stir bar sorptive extraction (SBSE) followed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. This method is based on the reaction of carvedilol enantiomers with (–)-menthyl chloroformate (MCF) after extraction by the SBSE method to produce diastereomeric derivatives. The separation was achieved by use of a C18 analytical column and the influence of mobile phase composition on the enantioseparation of carvedilol was studied. The applicability of two sorptive phases, poly(methyl methacrylate/ethylene glycol dimethacrylate) (PA–EG) and polydimethylsiloxane, were tested for extraction of carvedilol enantiomers from aqueous samples. The obtained results showed excellent linear dynamic ranges and precisions for each of them. The least limit of detection for (S)- and (R)-carvedilol obtained 8 and 11 µg L⁻¹, respectively, using the PA–EG sorptive phase. Inter- and intra-mean recoveries were also satisfactory, ranging from 98 to 103%, with coefficient of variation in the range of 1–5% at three fortified levels using a PA–EG coated stir bar. The proposed SBSE (PA–EG)–MCF derivatization–HPLC–UV method was successfully applied to enantioselective analysis of carvedilol in water and pharmaceutical dosages, confirming the application of this method.

Introduction
Separation of enantiomers from chiral medicines became a prominent topic in analytical chemistry, especially in the pharmaceutical and biological fields, because usually one enantiomer possesses very different and significant pharmacological, toxicological activities from the antipode and may even disturb other biological processes and cause catastrophic side effects (1, 2). Thus, the enantiomeric separation and analysis of chiral drugs have become essential in their monitoring and quality.

In recent years some significant advancements in separation methodologies in chiral chemistry have been achieved, which are modulations and variations of chromatography, and these advancements have paved the way for the quantitation of individual enantiomers of several racemates in biological fluids or the determination of enantiomeric excess of a variety of compounds in bulk drug or pharmaceutical formulations (3, 4). Among them, high-performance liquid chromatography (HPLC) has been successfully applied for the separation of enantiomers using chiral stationary phases without the requirement for any prior chiral derivatization (direct method), or using achiral stationary phases, which requires the formation of the diastereomers prior to chromatography (indirect method) (5, 6). The indirect method is sometimes simpler to perform and may produce better results than direct separation because chromatographic conditions can be optimized more easily. The main disadvantage of indirect methods, however, is interference of matrix in the derivatization step. As a result these methods require additional sample preparation steps to eliminate matrix effect, side-products or enriched target compound/s. In the majority of published methods for this purpose, liquid–liquid extraction (LLE) or solid-phase extraction (SPE) have been used (7). However, modern trends in analytical chemistry are toward simplification, miniaturization of sample preparation, and minimization of organic solvent used, and sample volumes.

Sorpptive micro-extraction techniques such as stir-bar sorptive extraction (SBSE) have become popular solventless approaches for the enrichment of priority organic compounds from food, environmental and biological aqueous matrices at trace level (8–11). The technique combines extraction and concentration of the analytes in a single step, thereby reducing the time required to prepare the samples. A problem associated with SBSE is the available SBSE coatings limitation because this analytical approach has been inspired in the use of polydimethylsiloxane (PDMS), which is a polymeric phase with higher affinity for nonpolar compounds. To overcome this problem, several authors have proposed new coatings, such as a glass fiber strip coated with three monolithic materials (12), polyurethane (13), polydimethylsiloxane–poly(vinylalcohol) (PDMS–PVA) (14), polyethylene glycol–polydimethylsiloxane–poly(vinyl alcohol) (PEG–PDMS–PVA) (15), polydimethylsiloxane–polypyrrole (PDMS–PP) (16), poly(methyl methacrylate–ethylene glycol dimethacrylate–acrylic acid) (PA–EG) (17, 18) and powdered activated carbon (19) to recover compounds with higher polarity.

Carvedilol, a currently relevant biologically active compound, is a nonselective β-adrenergic antagonist indicated in the treatment of hypertension, angina and congestive heart failure (20). Like other β-blockers, it is a chiral hydroxyl amine-containing compound, 1-(carbazolyl-4-oxy)-3-[2-(O-methoxyphenoxy ethyl) amino]-2-propanol. Although marketed as a racemic mixture, the β-receptor blocking activity of the (S)-enantiomer is ~200-fold higher than that of the (R)-enantiomer, whereas both enantiomers are equipotent α-blockers (21). Currently, the enantioselective quantification of carvedilol in pharmaceutical preparations or human plasma can be obtained by direct HPLC methods using a enantioselective column (22, 23), indirectly following a derivatization with an enantiomerically pure
reagent (24, 25), using a specialized electrophoretic method (26) or by sophisticated LC–MS-MS assays (27).

Herein, we report the application of SBSE followed by derivatization with (−)-menthyl chloroformate (MCF) as a chiral reagent for the enantiomeric excess determination of carvedilol using the HPLC method with UV detection. Extraction efficiencies of two sorptive phases PA–EG and PDMS were compared. All extractions were performed at optimum conditions according to our previous article (18). Finally, the performance of the proposed method was evaluated in terms of linearity, detection limits, accuracy and precision and the method was used in determination of carvedilol enantiomers in pharmaceutical dosage forms.

**Experimental**

**Materials**

Racemic carvedilol was obtained from Food and Drug Research Center (Tehran, Iran). The pure chiral agent MCF was purchased from Sigma (Steinheim, Germany). Methyl methacrylate (MMA), acrylic acid (AA), ethyleneglycol dimethacrylate (EGDMA), 2,2'-azobisis(2-methyl propionitrile) (AIBN, 98%), tris(hydroxymethyl) amino methane hydrochloride (Tris–HCl), sodium hydroxide, sodium acetate, toluene, chloroform, acetic acid and hydrochloric acid were obtained from Merck (Darmstadt, Germany). HPLC grade solvents used in the extraction procedure and for the mobile phase of the chromatographic system were prepared from Caledon (Georgetown, Canada). The water used throughout the study was purified using a Milli-Q water purification system (Millipore, St. Quentin, France). Two oral tablets containing 3.125 (Tablet I) and 6.25 mg (Tablet II) of each carvedilol enantiomer were obtained from a local drug store.

**Instruments and chromatographic conditions**

A HPLC system containing a Kontron Model 420 pump, a valve injector Rheodyne (Rohnert Park, CA, USA) equipped with a 20 µL loop and a Kontron Model 432 detector set to 285 nm was used with a Chromgate software (Knauer, Berlin, Germany) for acquisition system. The separation was performed in a C_{18} column (3 µm, 4.6 mm i.d. × 250 mm; Knauer, Berlin, Germany). The mobile phase was composed of methanol/acetate buffer (pH 4.0; 0.1 M) (70:30, v/v) in isocratic mode with a flow rate of 1.0 mL min⁻¹.

**Preparation of standard solutions**

The stock solution of racemic carvedilol was prepared in methanol at 1 mg mL⁻¹ concentration. This solution was then used to prepare aqueous solutions at concentrations of 20–2,000 ng racemic carvedilol mL⁻¹. The MCF solution was prepared daily in dichloromethane at 2% v/v concentration. An aqueous solution of 0.1 M sodium hydroxide was also prepared. For pharmaceutical dosage forms (Tablets I and II), ten tablets were powdered and an adequate amount of this powder was weighed and diluted in a volumetric flask using Tris–HCl buffer solution with pH 8.5.

**SBSE device**

The home-made stir bar coated with PA–EG film was prepared according to our previous work (17). The procedure of preparation, pretreatment, chemical modifications and coating of the glass bar were described completely in this reference. This procedure is a glass-encapsulated magnetic stir bar, externally coated with PA–EG. According to (17), the optimum composition for polymerization of PA–EG film on stir bar was 20% w/w EGDMA, 60% w/w MMA, and 20% w/w AA in the monomer mixture and 20% w/w porogen and 0.3% w/w AIBN in the total mixture. The SEM image of this film was shown in Figure 1. The used stir bar (11 mm length and 21 µL sorbent volume) was placed in a vial containing 1.0 mL of methanol and treated for 5 min with sonication between successive extractions. Then the solvent mixture was discarded and the procedure repeated three times. The stir bar was dried in a desiccator at room temperature.

The PDMS commercial Twister stir bar (with 10 mm length and 24 µL sorbent volume; purchased from Gerstel GmbH, Mulheim an der Ruhr, Germany) was also used. Prior to the first use, the stir bar was placed into a vial containing an acetonitrile–methanol solution (80:20; v/v) and conditioned for 24 h under agitation. Between successive extractions, the used stir bar was cleaned in methanol for 30 min at 50°C, under a magnetic stirring rate of 1,200 rpm, followed by a drying step using a lint-free tissue.

**SBSE and derivatization procedure**

The SBSE procedure was carried out by introducing either the PA–EG or the PDMA stir bars into 10 mL screw-cap vials and throughout the experiment these were stirred at constant speed (1,000 rpm). At the extraction step, 5 mL of the sample was stirred for a selected period of time at controlled temperature. At the desorption step, the stir bar was removed by using clean tweezers, rinsed slightly with MilliQ water (1.0 mL), dried with lint-free tissue and placed in a glass vial that containing 150 µL of the desorption solvent and assured that it is completely immersed. Desorption was performed by ultrasonic treatment during a selected period of time at controlled temperature. Then the desorption solvent was evaporated until dryness.
and 200 μL of 0.1 M sodium hydroxide aqueous solution and 200 μL of the chiral reagent MCF at 2% v/v concentration in dichloromethane were added to its residual. The mixture was sonicated for 5 min. Then 1 mL of water was added and the carvedilol diastereoisomers were extracted with 2 mL chloroform. After centrifuging the vial for 5 min at 3,000 rpm, the aqueous layer was discarded, and the remaining organic layer was evaporated until dryness (24). The obtained residues were dissolved in 150 μL of the mobile phase and 20 μL of it was injected into the HPLC system. The derivatization process for SBSE is shown in Scheme 1.

In our previous work (18), the optimal conditions for extraction of carvedilol by PA–EG and PDMS were obtained as follows: 5 mL sample solution adjusting at pH 8.5 stirred at 1,000 rpm and 50°C for 45 min. After the extraction, the analyte was desorbed in 150 μL methanolic solution of 1-methyl-3-octylimidazolium tetrafluoroborate ionic liquid ([Ommim][BF₄]) by ultrasonic agitation of the stir bar for 15 min at 45°C. But in this study pure methanol was used as desorption solvent to avoid occurring interference of ionic liquid for the derivatization step.

Method validation

For quantitative analysis using the proposed method, 0.5 mL of carvedilol standard solutions covering concentration ranges of 10–1,000 μg L⁻¹ for each enantiomer were diluted with 4.5 mL of buffer solution (pH 8.5) in a glass vial and extracted as described in the previous section. The calibration curves were constructed by plotting the peak area of each enantiomer against the respective concentration and the linearity was evaluated by the least-squares regression method, which was used to calculate the regression coefficient value (R), y-intercept and slope of the regression line. The limits of quantification (LOQs) as the lowest analyzed concentration and the limits of detection (LODs) as S/N = 3 were obtained for two enantiomers of carvedilol.

The accuracy and precision of the proposed method for both enantiomers of carvedilol were determined through analyzing standard quality control (QC) samples at three concentration levels of each carvedilol enantiomer (200, 325 and 425 ng mL⁻¹) for SBSE (PA–EG) and two concentration levels (325 and 425 μg mL⁻¹) for SBSE (PDMS). Accuracy was established by the recovery values (R, %) and precision was expressed in terms of coefficient of variation (CV, %) of the QC samples recoveries. Intra- and inter-day analyses were performed using three replicate extractions of each QC sample on 3 days.

In order to apply the proposed method for determination of carvedilol enantiomers in its two pharmaceutical dosage forms (declared Tablet I: 3.125 and Tablet II: 6.25 mg of each carvedilol enantiomer), four solutions containing equal amount of real sample and different amounts of carvedilol enantiomers standard solution (0, 250, 500, 750 and 1,000 μg L⁻¹) were prepared. Then according to the obtained optimum conditions carvedilol was extracted from these solutions, derivated and analyzed triplicate. Finally, the amounts of carvedilol enantiomers in real samples were calculated based on the standard addition method.

Results

Extraction of carvedilol using the SBSE method

Based on our previous research work (18), PA–EG and PDMS were employed as the SBSE adsorbent for the extraction of carvedilol from human serum. The experimental results showed that both sorptive phases could be effectively extracted when the pH of solution was 8.5. Also, 45 min as extraction time and 50°C as extraction temperature were employed for the extraction of carvedilol in subsequent experiments.

To investigate the extraction efficiency of carvedilol enantiomers by PA–EG and PDMS polymeric phases, at first, various concentrations of carvedilol (4–16 μg mL⁻¹) followed by derivatization with MCF were directly injected to HPLC, the calibration curves were constructed and the corresponding regression equations with the correlation coefficients higher than 0.993 and 0.995 were obtained for S- and R-carvedilol, respectively (data did not show). Then, the amount of the extracted materials from each sorptive phase was calculated through placing peak area of extracted ultra-pure water samples, containing 325 ng mL⁻¹ of each carvedilol enantiomer, in direct regression equations. Finally, the extraction efficiency was calculated as the ratio of the obtained amount of the extracted materials over the known amount of S- and R-carvedilol in aqueous samples (m_{extracted phase}/m_{0}). The results were obtained for both polymeric phases, in which extraction efficiency yields were 15.2 ± 1.4% (S-carvedilol) and 15.4 ± 1.5% (R-carvedilol) using a 24 μL PDMS stir bar and were increased about 2-fold to 32.6 ± 4.0%
(S-carvedilol) and 32.6 ± 3.2% (R-carvedilol) using a 21 μL PA–EG stir bar. This efficiency yields result in a lower LOQ values for aqueous extraction using PA–EG phase compared with the PDMS phase.

**Derivatization of carvedilol and separation of their diastereomers**

In the present investigation, MCF was used for derivatization of the carvedilol enantiomers. Derivatization reaction is shown in Scheme 1. MCF reacts with amines and alcohols and produce carbamate and carbonated derivatives, respectively; however, due to the presence of water, MCF reacts only with the amino group. Therefore, the carbamate derivatives formed can be extracted from the aqueous phase with an organic solvent at acidic or basic pH (24).

The mobile phase plays an important role in enantiomeric separation in terms of efficiency, retention and resolution of enantiomers. Therefore, investigation of the mobile phase composition should always be included in the optimization of enantioseparation conditions. Table I shows the effects of methanol and acetate buffer volume content in the mobile phase on the retention, selectivity and resolution of separated carvedilol enantiomers. All of these parameters increased with decreasing methanol volume content in mobile phase. Considering the retention time and the Rs value, a final amount of methanol of 70% (v/v) was selected for reduction of the analyzing time without compromising enantioselectivity.

Chromatograms of ± carvedilol aqueous solution (6 μg mL⁻¹) before and after derivatization with MCF are shown in Figure 2. In order to determine recovery of derivatization reaction, the area of carvedilol was compared with the total area of two carvedilol enantiomers. The recovery of derivatization reaction was obtained 84%.

**Validation of the proposed method**

The optimized SBSE followed by MCF derivatization and HPLC analysis was validated to develop an analytical method for the determination of carvedilol enantiomers in water and pharmaceutical dosage forms. The calibration equations, linear dynamic ranges, LODs and LOQs obtained for carvedilol enantiomers by both PA–EG and PDMS polymeric phases are presented in Table II. The results of this table indicate that the proposed methods provided adequate linearity for two enantiomers within the tested concentration intervals. The best sensitivities, LODs and LOQs for both enantiomers were obtained when PA–EG was used as sorptive phase. LODs (LOQs) were 8 (25) and 11 (50) μg L⁻¹ for S- and R-carvedilol, respectively.

The intra- and inter-day precision (% CV) were found to be <5% and accuracy ranged from 95 to 103% for SBSE (PA–EG)

<table>
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<th>Methanol/acetate buffer, pH 4, 0.1 M (v/v/v)</th>
<th>k₁</th>
<th>k₂</th>
<th>α</th>
<th>Rs</th>
</tr>
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<td>5.2</td>
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<td>12.5</td>
<td>14.7</td>
<td>1.18</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*(−)-Carvedilol.

*(+)-Carvedilol.

Figure 2. Chromatograms of racemic carvedilol solution (6.0 μg mL⁻¹) (A) before and (B) after MCF-derivatization using 70:30 v/v of methanol: acetate buffer (pH = 4, 0.1 M) as mobile phase and 1 mL min⁻¹ flow rate.
when determined at concentrations of 200, 325 and 425 \( \mu g \) L\(^{-1}\) of carvedilol enantiomers. For SBSE (PDMS), the intra- and inter-day precisions (\% CV) were found to be <8\% and accuracy ranged from 93 to 101\% (Table III). Based on statistical analysis, no significant differences were found between precision of PA–EG and PDMS polymeric phases.

### Analysis of real samples

Enantiomers of carvedilol were determined by carrying out recovery studies on two tablet dosage forms using the SBSE (PA–EG)–MCF derivatization–HPLC–UV method. Quantification analysis was performed using the standard addition method as mentioned in "Method validation" section. The precision of the extractions from tablet samples was examined by performing triplicate extractions. The obtained results are shown in Table IV. As can be seen, recoveries and CV values are between 96–104\% and 3–6\%, respectively.

### Discussion

The main purpose of this study is the use of new sorbent (PA–EG) for the SBSE method in order to increase the sensitivity compared with commercial sorbent (PDMS) such as previous our work (18) whereupon possibility of working in low concentrations has been achieved.

For separation of carvedilol enantiomers, MCF was used as chiral reagent. The MCF derivatization reaction is facile and the agent is inexpensive and readily available commercially. Based on the previous reports, MCF can be effectively used for the conversion of some \( \beta \)-blocker enantiomers to diastereoisomeric derivatives which are separated on standard reverse-phase columns (24). Based on the structure of produced carvedilol diastereomers (Scheme 1), the MCF derivatization introduced an additional aliphatic group to the carvedilol molecule. This structural change would enhance the hydrophobic interactions between analyte and \( C_{18} \) resulting in longer retention times for the derivatized compounds under the same mobile phase conditions. The obtained retention times confirmed this postulation (Figure 2). In agreement with previous studies, the sequence of elution was \( S(-) \) and \( R(+) \)-carvedilol (24).

In this study, the calibration curve of \( S(-) \) and \( R(+) \)-carvedilol was linear over the concentration range of 25–750 and 50–750 \( \mu g \) L\(^{-1}\), respectively, which is as good as or comparable with that reported in other article (28). The developed method was applied to the separation of carvedilol enantiomers in two commercial pharmaceutical dosages containing 6.25 and 12.5 mg of racemic mixture. No interference was observed in chromatogram due to the presence of excipients. The mean results for the determination of carvedilol enantiomers in both tablet dosage forms confirm the 50:50 enantiomeric mixtures of carvedilol using \( t \) test at 95\% confidence level.

### Conclusion

The SBSE–MCF derivatization–HPLC–UV analytical approach offers the opportunity of a practical and reliable methodology for the determination of the enantiomeric excess of carvedilol in pharmaceutical dosage forms. Under the optimized conditions a good analytical performance with excellent linear dynamic ranges and precisions was attained for the proposed method with a PA–EG and PDMS-coated stir bar. But home-made PA–EG sorptive phase was shown recovery yields much better than the conventional PDMS and the least limit of detections for the analyte under study. Using the standard addition method, the established methodology showed excellent response for the determination of the enantiomeric excess of carvedilol in tablet dosage forms. Also, application of this method can be extended to determine carvedilol enantiomers in biological fluids due to possibility of using the SBSE method in biological matrices.
Acknowledgments
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