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# A dispersive liquid–liquid microextraction and chiral separation of carvedilol in human plasma using capillary electrophoresis

**Background:** Development of simple, rapid and precise analysis of chiral drugs in biological samples is an important issue. Dispersive liquid–liquid microextraction in combination with CE using field amplified sample injection has been of interest because of its capability to analyze trace amount of drugs. **Methods:** Dispersive liquid–liquid microextraction-CE-field amplified sample injection was employed for chiral separation of carvedilol in human plasma using UV-DAD detector and the developed method has been validated according to US FDA method validation guideline for bioanalysis. **Results:** The method was linear over a concentration range of 12.5–100 ng/ml for each carvedilol enantiomer ( $R^2 = 0.998$ ) and the mean recoveries ranged from 91 to 107%. **Conclusion:** The method was adapted for sensitive, selective and rapid determination of carvedilol enantiomers in human plasma samples.

Although the merit of chirality has been known for decades, the pharmaceutical significance of racemic drugs has only been widely recognized in the last decades [1–3]. Realizing the role of chirality in pharmacology becomes more crucial following the thalidomide tragedy in 1960s. Enantiomers may show similar or different pharmacological effects. Characterizing the contribution of stereochemistry in a drug's action is important to develop more effective and safer drugs.

There is a high demand for enantioseparation at analytical scale for **therapeutic drug monitoring** (TDM), optimization of pharmacotherapy, pharmacokinetic and pharmacodynamic studies. Furthermore, as certain enantiomers represent illicit or banned substances, chiral discrimination shows an important challenge in forensic analysis and doping control [4,5].

**Carvedilol** ( $\pm$ )-[3-(9H-carbazol-4-yloxy)-2-hydroxypropyl][2-(2-methoxyphenoxy)ethyl] amine (CAR) (molecular structure, therapeutic level, logP and  $pK_a$  are summarized in **Table 1**) is a nonselective  $\beta$ -blocker/ $\alpha_1$ -blocker which indicated in the treatment of heart failure and hypertension as a racemic mixture.

Physicochemical properties calculated using ACD/labs software (ACD/Structure Elucidator, Advanced Chemistry Development, Inc., Toronto, ON, Canada [7]).

CAR acts by blocking adrenergic receptors in blood vessels and cardia which result in its hypertensive and bradycardic effects. It works by relaxing blood vessels and decreasing the heart rate to improve blood flow and decrease blood pressure. It is also important because of its antioxidant effects, neutrophil infiltration reduction, apoptosis inhibition, reduction of vascular smooth muscle migration and improvement of myocardial remodeling post acute myocardial infarction [8]. Additionally, CAR is a prohibited agent according to the World Anti Doping Agency (WADA).

CAR enantiomers exert different pharmacological responses: it exerts a similar effect on  $\alpha_1$  adrenergic receptors; however, the (*S*)-CAR is attributed to  $\beta$ -blockade activity (50- to 500-times higher than (*R*)-CAR) [9]. In addition to different pharmacological action, each enantiomer exhibits unique plasma concentration, bioavailability and protein binding. Therefore, it is vital to determine the concentrations of each CAR enantiomer in biological fluids [10–13].

Samin Hamidi<sup>1</sup>, Somaieh Soltani<sup>2</sup> & Abolghasem Jouyban<sup>\*1</sup>

<sup>1</sup>Drug Applied Research Center & Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz 51664, Iran

<sup>2</sup>Liver & Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz 51664, Iran

\*Author for correspondence:

Tel.: +98 41 33379323

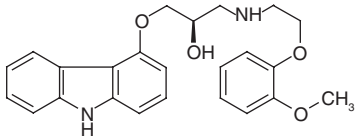
Fax: +98 41 33363231

[ajouyban@hotmail.com](mailto:ajouyban@hotmail.com)

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Table 1. Molecular structure and physicochemical parameters of carvedilol†.

	
Therapeutic range (ng/ml)	20–160 [6]
Log P	4.11
pK <sub>a</sub>	7.5
†Indicates the chiral center.	

Several enantioseparation methods of CAR using HPLC in human plasma or blood have been reported accompanied by indirect precolumn chiral derivatizing agents or direct method coupling with various columns [14–19].

CE has been proven as a separation tool in enantioseparation of different chiral compounds. The enantioseparation of CAR using CE has been reported in some studies and the summary of the results are listed in [Supplementary Table 1](#).

In comparison with HPLC and GC, CE exhibits some advantages in chiral separation including: flexibility by the use of various chiral selectors, higher resolution power, separation efficiency, various separation modes and low solvent and/or reagents consumption. Enantioseparation in CE by addition of a chiral selector to background electrolyte (BGE) is much simpler than expensive chiral stationary-phase

### Key terms

**Therapeutic drug monitoring:** Measurement of drug concentrations in biological samples for adjustment of drug regimen in personalized medicine.

**Carvedilol:** Nonselective  $\beta$ -blocker/ $\alpha$ 1-blocker for management of heart failure and hypertension as a racemic mixture.

**CE:** CE is an instrument for separation of analytes based on their electromigration behavior. In this work, we used CZE mode modified by cyclodextrin for chiral separation of carvedilol.

**Clean up:** Pretreatments on samples for separation of analytes from interfering compounds of the matrix.

**Field-amplified sample injection:** Powerful on-line sample preconcentration method that improves the detection sensitivity by using the conductivity difference between sample zone and the background electrolyte in CE.

**Dispersive liquid–liquid microextraction:** Rapid injection of a mixture of extraction and dispersive solvents into an aqueous sample solution, resulting in cloudy mixture of very tiny droplets of extraction solvent fully dispersed in the aqueous phase.

columns in HPLC or even laborious precolumn indirect derivatization methods in GC. Most commercial CE instruments are coupled with popular UV detector that inherently limits the sensitive detection of samples in low concentrations. Therefore, its detection sensitivity has been shadowed by small injected sample volume (2–10 nl) as well as short optical path length of detector window [20]. In addition to these limitations, the complexity of biological samples further confines the detection of drugs in biological fluids.

Optical sensitivity can be improved by modifying capillary detection window for UV detection. Bubble cells or Z-type cells are the examples of extended light path. More sensitive detectors (such as fluorescence and electrochemical) could be replaced with low sensitive UV detector [21]. Preconcentration methods have been developed to improve S/N which are important for complicated matrices such as biological samples. They are divided into on-line and off-line techniques. On-line preconcentration methods are based on velocity mismatch between sample zone and BGE or based on the chromatographic principle [20]. Variable methods have been utilized for this purpose, namely transient isotachopheresis ( $t$ -ITP) [22] dynamic pH junction [23], sweeping [24], large volume sample stacking (LVSS) [25] and field amplified sample stacking (FASS) [26]. FASS depends on conductivity differences between sample zone and BGE. The effect of conductivity difference on sensitivity enhancement was first explained by Mikkers *et al.* in 1979 [27]. Generally, the ions slowed down during the migration through the low conductivity region toward high-conductivity region. Thus, the narrow boundary zone between sample plug and buffer was stacked resulting in more sensitive detection. Extraction and **clean up** from biological samples prior to CE analysis is an important issue in FASS. The principle of **field amplified sample injection** (FASI) is similar to FASS. The only difference is in the injection mode, which is caused by different focusing process. In FASS, a hydrodynamic injection is applied and stacking occurs with applying the voltage. In FASI mode, a sample is loaded with electrokinetic injection, therefore the focusing process occurs at the beginning of the injection. The efficiency of FASS as well as FASI suffers from matrix effect in real biological samples. Thus, application of suitable sample treatment methods (off-line techniques) should be regarded.

Consumption of hazardous solvents and costly consumables of traditional extraction methods, such as liquid–liquid extraction (LLE) and SPE are among the most important disadvantages of these methods and

attempts to overcome such problems were addressed toward miniaturization of solvents and application of alternative materials. Liquid phase microextraction (LPME) was one of the feasible methods developed in this regard and its lower cost, excellent clean up capability and possibility of low solvent consumption resulted in frequent applications in pharmaceutical and biomedical analyses [28,29]. **Dispersive liquid–liquid microextraction** (DLLME), which was introduced by Rezaee *et al.* in 2006 [30], is one of the most frequently studied LPME techniques because of its ability to enhance the effective interaction of analyte with extraction solvent. Briefly, a mixture of dispersive and extraction solvents are rapidly injected (within 3 s) into a sample solution to form very tiny droplets. The extraction time is shortened due to the formation of infinitely large contact surface between the extractant droplets and liquid phase. The details for application of DLLME combined with CE are tabulated in **Supplementary Table 2**. According to the table, the developed methods use traditional extraction methods, and the application of DLLME in enantioseparation of CAR has not been studied yet. Reports of the application of DLLME-CE method in enantioseparation were published in 2011 and 2013, studying the enantioseparation of multiple illicit drugs in forensic samples (such as banknote, kraft paper, plastic bag and silver paper) [31] and chiral biotransformations [32], respectively.

In this study, DLLME procedure is performed for clean up and CE-DAD was used for chiral separation of CAR in human plasma samples and FASI methods are used to enhance the sensitivity. Effects of different variables on chiral separation (e.g., concentration of chiral selector, buffer concentration and pH) and DLLME performance (e.g., type and volume of extraction and dispersive solvents and pH) were studied. The proposed method is validated according to FDA guideline and its applicability for determination of concentrations of CAR enantiomers is shown in real plasma samples collected from two patients.

### Chemicals & solutions

CAR powder was purchased from SalehanChemical Company (Tehran, Iran). (*R*)- and (*S*)-CAR enantiomers were purchased from Toronto Research Company (Toronto, Canada). Acetonitrile (ACN), methanol, acetone and chloroform (CHCl<sub>3</sub>) were obtained from Scharlau (Barcelona, Spain). Sodium hydroxide, orthophosphoric acid, carbon tetrachloride (CCl<sub>4</sub>), dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), 1,2-dichlorobenzene (1,2-DCB), triethanolamine (TEA), dimethylformamide (DMF), formamide, 1-propanol and hydrochloric acid were purchased

from Merck (Darmstadt, Germany). Carboxymethyl- $\beta$ -cyclodextrin (CM- $\beta$ -CD) was purchased from Fluka Chemical (Buchs, Switzerland). All reagents and solvents were of analytical grades. Deionized water (Shahid Ghazi Company, Tabriz, Iran) was used for sample and BGE preparations.

### Instrumentation & CE condition

A CE instrument (Agilent Technologies 7100 [Waldbronn, Germany]) equipped with a diode array detector (DAD) (190–600 nm) was used for analysis. DAD is a sophisticated version of UV detectors with the capability of scanning UV spectrum of a separated peak at a given wavelength range. Agilent Chemstation® (Waldbronn, Germany) software was applied for data analysis and instrument control. The proportional height as well as area of the individual peak, were calculated using Chemstation software. Separation was accomplished on bare fused silica capillaries (50 cm length, 41.5 cm effective length and 50  $\mu$ m id) were purchased from Agilent Technology (Waldbronn, Germany). Each new capillary was washed with sodium hydroxide (1.0 M) for 1 h, deionized water (1 h) and BGE for 1 h. Between each run, capillary was flushed for 3 min with sodium hydroxide (0.1 M) and 3 min with deionized water then conditioned by BGE for 5 min. The BGE composition was 100 mM phosphoric acid-TEA at pH 2.5 containing of 30% (v/v) methanol and 0.6% (w/v) CM- $\beta$ -CD. All samples were injected at 15 kV for 30 s after the loading a short water plug (50 mbar  $\times$  1 s). The applied voltage was 25 kV and the temperature was 15°C. Detection was performed at 241 nm and pH adjustments were made by a Metrohm® pH meter (Herisau, Switzerland). Hettich centrifuge (Tuttlingen, Germany) was used for sedimentation of the extraction solvent in sample preparation. The vortex from Labtron Company (Tehran, Iran) was used in sample preparation.

### Standard solutions & plasma samples

Stock standard solution of CAR (1000 mg/l) was prepared in methanol and stored at 4°C. For preparation of daily standard solutions, the appropriate volume of stock solution was diluted with methanol.

Drug-free QC plasma samples were provided by Blood Transfusion Research Center (Tabriz, Iran) and transferred in propylene microtubes and frozen at -4°C. Plasma samples thawed at room temperature before daily experiments. In order to prepare the desired concentration of CAR in plasma, appropriate amount of racemic CAR standard solution was spiked into 0.5 ml drug-free plasma. Sample preparation is the key step in complex biological fluids in limited amounts. Protein precipitation step performed by adding 1.0 ml acetone.

1.0 ml of supernatant was collected after centrifuging at 3461 g for 10 min and transferred to the 10 ml glass conical button tube followed by the addition of 8.0 ml NaOH solution (pH 10.0). Two samples were obtained from patients receiving CAR who had signed consent forms approved by the ethics committee, Tabriz University of Medical Sciences. Samples were collected in polypropylene tubes and stored at  $-20^{\circ}\text{C}$  until analysis.

### DLLME procedure

After preparation of plasma sample, the mixture of the extraction/dispersive solvents (100/500  $\mu\text{l}$ , respectively) was rapidly injected into the sample tube using a 2.0 ml syringe. A cloudy mixture quickly formed and the emulsion was centrifuged at 1730 g for 7 min to obtain the sedimented phase. The aqueous phase carefully separated and then collected sediment was transferred into a microtube and dried under a gentle stream of  $\text{N}_2$  for 10 min. Subsequently, the residue was reconstituted in 100  $\mu\text{l}$  of water/acetonitrile (1:1) mixture and vortexed for a few seconds and then introduced by electrokinetic injection into CE-DAD for analysis. Schematic diagram of the proposed DLLME is shown in Figure 1.

### Method validation

Partial bioanalytical method validation was performed according to FDA guideline [33]. The calibration linearity, LOD, LLOQ, ULOQ (lower and upper LOQ, respectively), intra- and interday precisions, accuracy, recovery, selectivity, stability (room temperature and freeze-thaw) and robustness were tested. The application of the developed method in clinical samples was tested using two patient samples.

## Results & discussion

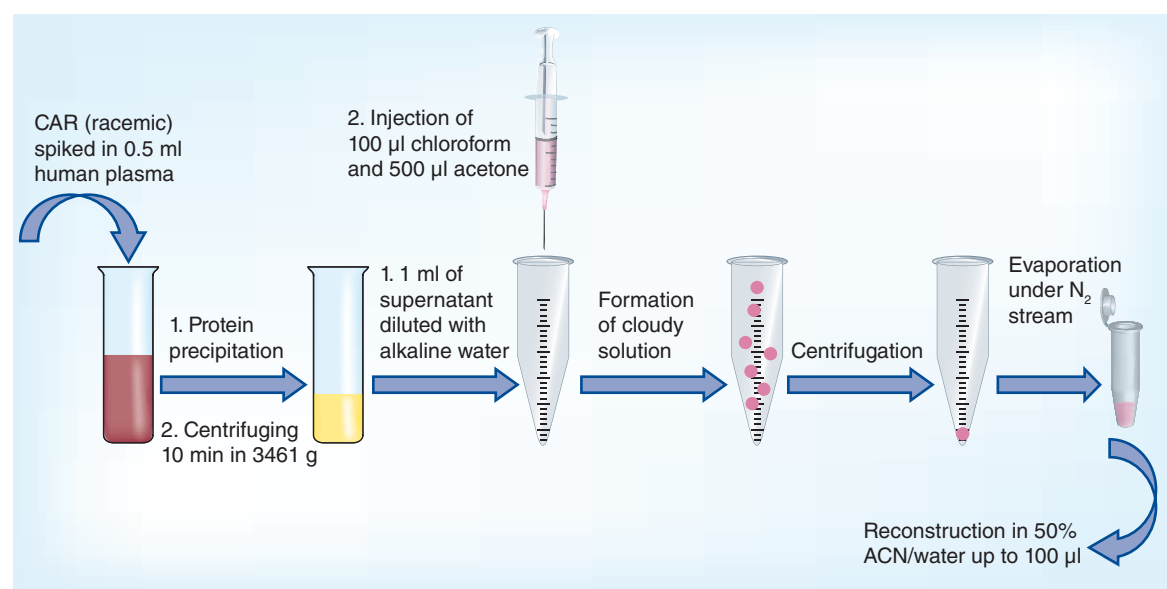
Preliminary studies target the influence of several parameters including concentration of chiral selector, buffer concentration, pH, applied voltage, temperature and organic modifier on resolution. In this work, CM- $\beta$ -CD as an inexpensive chiral selector evaluated for enantioseparation of CAR with CE. Although, neutral and charged  $\beta$ -CDs were previously studied by some authors for enantioseparation of CAR (see Supplementary Table 1 for details), this is the first report of the application of CM- $\beta$ -CD as a chiral selector in enantioseparation of CAR.

### CM- $\beta$ -CD concentration optimization

A total of 100 mM phosphoric acid-TEA buffer (pH 2.5) containing 30% (v/v) methanol with six different amounts (0–1% [w/v]) of the CM- $\beta$ -CD were introduced to the CE and the resolutions were plotted against CM- $\beta$ -CD concentration (Figure 2). Maximum resolution ( $R_s = 4$ ) belongs to 0.6% (w/v) CM- $\beta$ -CD. At the higher concentrations (> 0.6% [w/v]) the resolution decreased because of saturated state between the enantiomers and chiral selector. It is coincidence with Wren and Rowe model, which states that maximum resolution occurred at a certain concentration of a chiral selector [34]. Accordingly, the 0.6% (w/v) of selector was chosen for subsequent steps.

### Buffer concentration

Electroosmotic flow (EOF), Joule heating and the adsorption of analyte on the inner surface of capillary column, are the phenomena which were influenced by buffer concentration [35]. The 0.6% (w/v) CM- $\beta$ -CD



**Figure 1. Operating procedure in dispersive liquid-liquid microextraction.** ACN: Acetonitrile; CAR: Carvedilol.

and 30% (v/v) methanol at pH 2.5 BGE were applied for the optimization of phosphoric acid–TEA buffer (30–100 mM) concentration. By increasing of the phosphoric acid–TEA concentration from 30 to 100 mM and the consequent buffer ionic strength enhancement, a corresponding increase in resolution was observed. An increase in resolution can be attributed to decrease in EOF therefore, adsorption the analyte to inner surface of capillary decreased. The concentrations above 100 mM were avoided due to unfavorable baseline separation.

### pH optimization

With an increase in pH from 2.5 to 3.2 the resolution is declined. Chiral selector is in its neutral form at low pH ( $pK_a$  4.36) and CAR is fully protonated. Baseline resolution of the peaks along with acceptable peak shapes was achieved at pH 2.5. Decreasing the resolution at higher pH may be as a result of weak complexation of CAR with the chiral selector.

### Temperature optimization

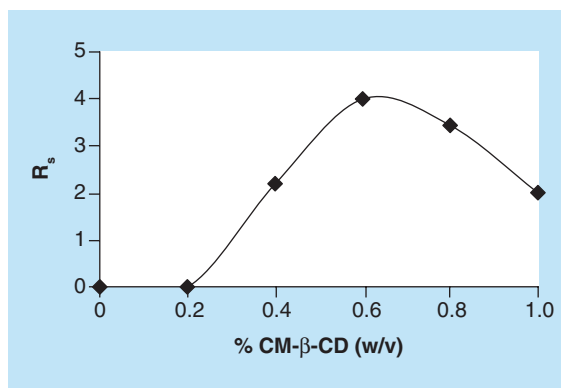
Temperature variations affect enantioseparation with different mechanisms, for example, buffer viscosity, Joule heating, migration times and complex formation equilibria between enantiomers and chiral selector [35,36]. Several capillary column temperatures (15, 20 and 25°C) were checked. At the temperatures higher than 15°C, resolution dramatically decreased ( $\sim 0$ ) which was possibly linked to viscosity changes and/or thermodynamic behavior of the analyte and selector complex.

### Separation voltage

Interactions between selector and selectant also influenced by variation of the separation voltage. Joule heating and the electrophoretic velocities of EOF and the analyte, are the parameters that could be subjected on separation. Higher voltage brought instability in baseline as well as poor separation. Generation of excessive Joule heating explained these phenomena [37]. Viscosity of the buffer decreases when heat rises inside the capillary column resulting in unsatisfactory resolution. Different voltages (20–30 kV) were applied using 100 mM phosphoric acid–TEA buffer (pH 2.5, 0.6% w/v CM- $\beta$ -CD and 30% methanol) and baseline separation and more stable baseline was obtained at 25 kV ( $I \approx 21 \mu\text{A}$ ). At higher voltages the electrical current shows low stability.

### Organic modifier

Different proportions of methanol (0–30% [v/v]) were added to the BGE (other parameters were in optimized condition) and the results showed that the resolution was zero in the absence of methanol and the enantiomers were completely separated in 30% methanol. This



**Figure 2.** The effect of chiral selector concentration on the resolution of carvedilol enantiomers.

phenomenon described by Wren and Rowe and could be explained by changes in binding constants (hence separation) of enantiomers and the selector [38].

### Optimization of DLLME parameters

The details of optimized parameters (type and volume of extraction and disperser solvents and pH) discussed following topics. The spiked concentration of racemic CAR was 0.2  $\mu\text{g}/\text{ml}$  and all the experiments were performed in QC plasma samples.

### Extraction solvent type

The aim of optimization of extraction solvent is to improve the extraction efficiency. Extraction solvent should cover following requirements: denser than water, low solubility in water, suitable for CE, high extraction efficiency, no interferences with the analyte peak during separation [39,40] and ease of evaporation. The extraction solvent should be selective for targeted analyte and not for unwanted compounds which exist in the matrix. Sometimes impurities overlap with the analyte peak in CE which could be eliminated using a selective extraction solvent. Due to the incompatibility of halogenated solvents (solvents denser than water) with capillary columns, and necessity of solvent evaporation and reconstitution in a compatible solvent, ease of solvent evaporation should be regarded. Concerning the mentioned criteria some chlorinated solvents such as  $\text{CHCl}_3$ , 1,2-DCB,  $\text{CCl}_4$  and  $\text{CH}_2\text{Cl}_2$  were examined by applying 60  $\mu\text{l}$  of each solvent to 1000  $\mu\text{l}$  of acetonitrile as a dispersive solvent. In the case of  $\text{CH}_2\text{Cl}_2$  and  $\text{CCl}_4$ , the amount of settled phase was low due to their solubility in water. With respect to better baseline separation, resolution and maximum signal,  $\text{CHCl}_3$  was employed for future studies.

### Extraction solvent volume

Different volumes of extraction solvent (i.e., 70, 80, 90, 100 and 120  $\mu\text{l}$ ) were tested. Volumes lower than

70  $\mu\text{l}$ , were not used due to the handling problems. Higher peak area was observed for 100  $\mu\text{l}$ .

### Dispersive solvent type & volume

Dispersive solvent in DLLME should be miscible with both extraction solvent and sample solution. In addition the volume of dispersive solvent is the key factor in DLLME. In order to choose most suitable solvent, methanol, acetone and ACN were investigated. In the presence of methanol and chloroform the tiny droplets did not form well and no phase separation is observed. Acetone (compared with ACN) showed highest peak area. On the other hand, selection of acetone was convenient with protein precipitating solvent (acetone). To figure out the suitable dispersive solvent volume, experiments were performed with different volumes of acetone (400, 500, 600 and 700  $\mu\text{l}$ ) introducing with 100  $\mu\text{l}$   $\text{CHCl}_3$ . 500  $\mu\text{l}$  acetone was selected as an optimum volume.

### pH

Chloroform was found as an excellent extraction solvent, but its reactivity with bases reduces its utilization with basic drugs such as CAR that need to be extracted at high pH. This subject encouraged the authors to investigate the extraction in wide range of pHs. Effect of pH on the extraction of CAR was adjusted in the range of 3.0 to 12.0 using NaOH (1.0 M) or HCl (1.0 M). At the basic pH, CAR is in its uncharged form so it could be readily extracted within  $\text{CHCl}_3$  droplets. In the case of pH 10, the organic phase was clearer than other pHs. So, we rather selected pH 10 for subsequent analysis.

### Biologic matrix effect on DLLME

Without protein precipitation and dilution steps the distinct sedimented phase was not achievable. Volume of sample solution varied from 5.0 to 10.0 ml while the volume of spiking plasma (after protein precipitation) fixed at 1.0 ml. According to our findings below five-times dilution the white sedimented phase resulted, which diminished the efficiency of extraction. Finally, primary protein precipitation following nine-times dilution was selected as a pretreatment step for samples.

### FASI optimization

The nature of solvent for redissolving dried analyte, water plug injection time, sample plug injection time and voltage were investigated to improve the sensitivity. The mobility of analyte has been affected by dielectric constant and the viscosity of the sample solvent [41]. At higher mobility, the amount of injected sample is increased. In this work dissolving the sample

in BGE media (for conventional injection) or diluted buffer (at least ten-times) which was one of the necessity of stacking, did not result in satisfactory results. Reconstitution of DLLME extracted sample were checked using water and low conductivity binary mixtures of water: methanol, propanol, ACN, DMF and formamide over the range of 0–100% (v/v). The highest peak area resulted from the 50% ACN (Figure 3A) and further ACN caused peak broadening. Concerning the results, water + ACN (1:1) was chosen as CAR redissolving solvent.

Electrokinetic injection performed due to its applicability for on-line preconcentration. The effect of injection time and voltage were optimized by applying voltage and injection time varied from 10 to 20 kV and 20 to 35 s, respectively. Varying the voltage up to 15 kV was increased the peak area, but further increase in voltage resulted in peak broadening. Results are summarized in Figure 3B. Sample plug injected by applying 15 kV for 30 s.

By introducing a low conductivity preinjection plug prior sample plug, the electric field strengthened at the beginning of the injection [42]. A preinjection water plug is loaded at 50 mbar for 0 to 5 s. Exceeding the time of injection over 1 s, caused to disrupting the resolution. Water plug was injected at 50 mbar for 1 s help to slightly increase the signal.

### Method validation

#### Calibration curve & linearity

The average of three replicated calibration curves in three different days, was used for validation studies. The calibration curves constructed by plotting the signal against the ascending concentration of CAR, were linear in the range of 12.5 to 100 ng/ml for each enantiomer ( $R^2 = 0.998$ ). The standard deviation values for slope and intercept were reported for each enantiomer after constructing the calibration curves using three separate runs. The details of calibration curves, LOD, LLOQ and ULOQ were tabulated in Table 2. The resulted linear range covers the therapeutic range of CAR (20–160 ng/ml), if the found concentration is above 100 ng/ml, a simple dilution step is required.

$\text{LOD} = 3 \times (\text{Sb}/a)$  where Sb is the standard error of intercept and a is the slope of calibration curve.

#### Precision & accuracy

Inter- and intraday precisions and accuracies were checked by determination of three different concentrations by analyzing QC samples for five-times. According to the FDA guideline the %RSD values lower than 20% for LLOQ and 15% for other

calibration samples are acceptable for bioanalysis. The results (reported in [Supplementary Table 3](#)) indicated that the mean RSD% for all inter and intraday assays were seven and 6%, respectively. While the highest RSD% was detected for 25 ng/ml sample that was in an acceptable range. Accuracy of method was defined as relative error (RE%) calculated using following equation:

$$\text{RE\%} = 100 \times \left( \frac{\text{Found value} - \text{Nominal value}}{\text{Nominal value}} \right)$$

Equation 1

Mean RE% for all inter and intraday assays were 1 and 0.2, respectively and the highest RE% was detected for 25 ng/ml sample which was within an acceptable range.

### Recovery

QC samples were spiked with three different concentrations of racemic CAR and subjected to protein precipitation and DLLME procedure. The relative recoveries (RR%) being defined as the measured concentration of the drug divided by its actual concentration ([Table 3](#)). Mean recovery for all samples was 100.2% and the most negative and positive deviated recoveries were reported for 25 and 50 ng/ml, respectively, which were acceptable according to the guideline [33].

### Stability

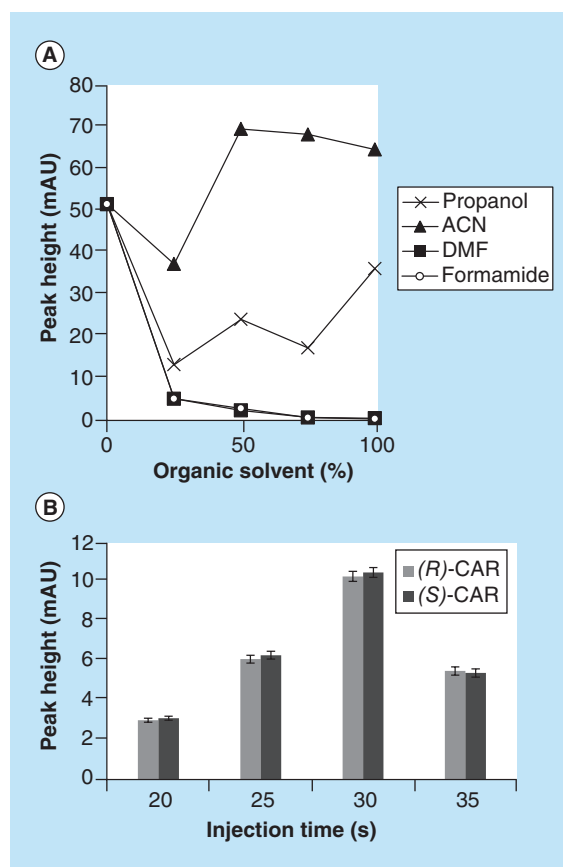
For investigating the stability of CAR in biological fluids, the analysis performed after 12 h at room temperature and over three freeze-thaw cycles. The results ([Supplementary Table 4](#)) revealed no significant instability for the studied samples.

### Selectivity & specificity

Availability of any probable interference with CAR peaks studied by injecting different drug-free human plasma. As shown in [Figure 4A](#), no interference peak observed from plasma matrix. In order to investigate the selectivity of method, 0.5 µg/ml of different probable co-administered drugs or similar β-blockers (losartan, sotalol, diltiazim, salicylic acid, verapamil, atenolol, diazepam, amiodarone, amiloride, nifedipine, acetaminophen, furoside, hydrochlorothiazide and propranolol) were extracted using DLLME and injected to CE using the same condition. No overlapped peaks were observed.

### Robustness

The probable sources of variability in operating procedure estimated by slight variation of some



**Figure 3. Optimization of the injection parameters for field amplified sample injection. (A)** Effects of solvent compositions of sample plug on sensitivity in FASI mode; 1 µg/ml of CAR assayed in binary mixture of water and various organic solvents ( $n = 1$ ) and **(B)** Sample injection time for 200 ng/ml CAR enantiomers in 50% ACN which was obtained using dispersive liquid–liquid microextraction for QC plasma. The separation conditions are: 100 mM phosphoric acid–triethanolamine buffer at pH 2.5 containing 30% (v/v) methanol and 0.6% (w/v) CM-β-CD. The applied voltage is 25 kV. The detection wavelength is 241 nm, the capillary is 50.0 cm (effective length of 41.5 cm) × 50 µm id, and the system temperature is 15°C. The error bars indicates standard deviations ( $n = 3$ ). ACN: Acetonitrile; CAR: Carvedilol; DMF: Dimethylformamide.

parameters by spiking 200 ng/ml of racemic CAR in QC samples followed by DLLME and consequent CE analysis. Slight variation of some instrumental parameters such as buffer pH (pH 2.45, 2.50 and 2.55), capillary column temperature (14.5, 15.0 and 15.5°C), and applied voltage (24.5, 25.0 and 25.5 kV) were studied. Negligible variation in peak area, migration time and resolution confirmed the reliable robustness of the method. To judge the robustness of the sample preparation, influence of significant parameters on DLLME was checked. Variation the pH (i.e., pH ±1) revealed that the procedure was robust in a relatively

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

Compound	Slope	Slope standard deviations (n = 3)	Intercept	Intercept standard deviations (n = 3)	R <sup>2</sup> value <sup>†</sup>	Linear range <sup>‡</sup>	LOD <sup>‡</sup>	LLOQ <sup>‡</sup>	ULOQ <sup>‡</sup>
(R)- CAR	0.076	0.0004	1.466	0.03	0.998	12.5–100	4	12.5	100
(S)- CAR	0.074	0.0002	1.491	0.03	0.998	12.5–100	4	12.5	100

LOD = 3 × (Sb/a) where Sb is the standard error of intercept and a is the slope of calibration curve.

<sup>†</sup>Coefficient of determination

<sup>‡</sup>Concentrations are based on ng/ml.

the wide pH range. Furthermore, the variation of centrifuge time and rate had no effect on the DLLME performance (see Supplementary Table 5 for details).

### Application to clinical samples

In order to investigate the applicability of developed and validated method for determination of CAR enantiomers in real plasma samples, final experiments were operated on two patients samples. Details of the patients along with levels of (R)- and (S)- CAR in these plasma samples are summarized in Table 4. The drug quantification was calculated using the calibration curve. Also electropherograms of spiked sample as well as one patient sample (patient 2) are shown in Figure 4B & C, respectively. Each CAR enantiomer typically has different concentrations in biological fluids [10–13]. Detailed discussion about this needs extensive study on more samples which is not in the objective of this work.

According to the obtained results and concerning therapeutic concentration of CAR, in other words, 20–160 ng/ml [6] and coverage of this level by the method, its simplicity and also capability for routine analysis, the method could be proposed for practical application.

### Comparison of the proposed method with others

Supplementary Table 1 summarizes linear range, LOD, LOQ, matrix type and validation of some analytical methods along with the proposed method. Several methods have been reported for CAR enantiomers

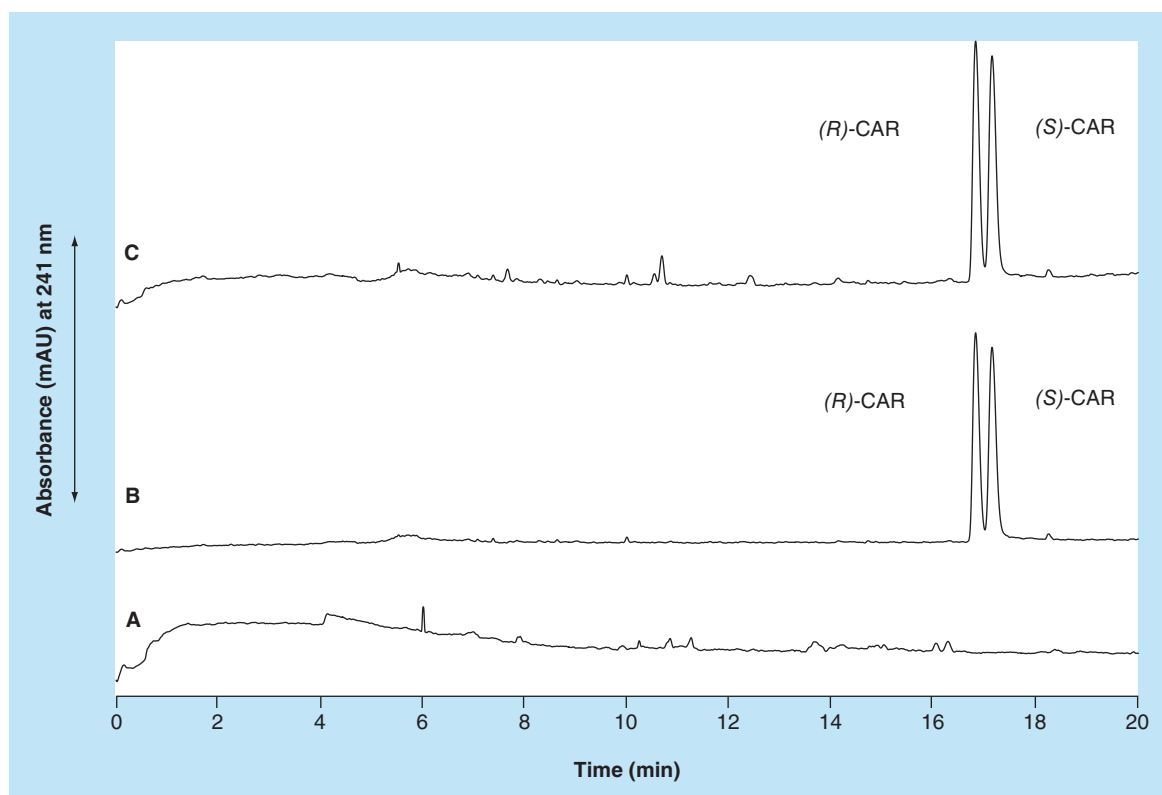
determination in biological fluids using CE, so far. Protein precipitation method and LLE were employed in the most cases for clean up or preconcentration that involve time consuming and extensive sample preparation procedures before instrumental analysis. In most cases, which were used commercially available UV detector, the resulting LODs were significantly more than that of the proposed method. Proposed DLLME presents very low LODs values in comparison with methods which were used inherently low sensitive UV detectors. It should be noted that in one case [11] lower LODs values were achieved using LIF detector which is more sensitive than UV. Although dynamic linear range of the proposed method is not as wide as the others, but LLOQs are much lower to cover the therapeutic purposes. In the samples with high concentrations, with simple dilution step desired concentration will be attained. In order to minimize the consumption of hazardous solvents, efficient miniaturizing performance is desirable. Proposed method is rapid, simple, inexpensive and sensitive enough for CAR enantiomers monitoring in plasma. Furthermore, developed method was validated according to FDA guideline.

### Conclusion

A new, cost-effective and simple clean up DLLME procedure was developed and validated for the extraction of CAR from human plasma. On the contrary of traditional methods which consumed large amounts of expensive and hazardous solvents, DLLME carried out just by adding a few microliters of a high-density extraction solvent (100 µl chloroform) and a dispersive

Table 3. Recoveries for extraction and analysis of carvedilol in spiked plasma samples with dispersive liquid–liquid microextraction-CE-DAD.

Analyte	Nominal concentration (ng/ml; n = 5)	Mean found concentration (ng/ml)	Mean recovery (%)
(R)- CAR	25	23	95
	50	52	105
	100	99	99
(S)- CAR	25	22	91
	50	53	107
	100	103	103



**Figure 4. Typical electropherograms of carvedilol.** In (A) drug-free plasma sample (B) 60 ng/ml (racemic) spiked plasma sample and (C) patient (2) sample. CAR: Carvedilol.

solvent (acetone) to the plasma sample. It helps to shorten the extraction time and facilitate the extraction efficiency owing to form the very tiny droplets in few seconds. DLLME eliminates the matrix effect. The extracted samples were analyzed using CE-DAD with the application of CM- $\beta$ -CD as a chiral selector for the first time. The proposed DLLME-CE-DAD procedure provided precise and accurate results and it could be applied for the clinical aims especially TDM of CAR enantiomers, pharmacokinetic, pharmacodynamic and forensic studies.

### Future perspective

The use of DLLME-CE technique for the analysis of enantiomers of drugs in various biological media will supply quick determination of enantiomers. DLLME provides excellent clean up which helps to remove the

interfering compounds from complicated biological media and this capability could apply for trace analysis of metabolites and biomarkers using CE or other separation methods.

### Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: [www.future-science.com/doi/full/10.4155/BIO.15.51](http://www.future-science.com/doi/full/10.4155/BIO.15.51)

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**Table 4. Some details of the real samples analyzed for carvedilol enantiomers by the proposed method.**

No.	Gender	Age	Intake time (AM)	Sampling time (PM)	R (ng/ml)	S (ng/ml)
1	Male	62	10	5	64	65
2	Male	50	9	6	36	33

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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

##### Method development & validation

- A simple and rapid dispersive liquid–liquid microextraction-CE-field amplified sample injection was employed for chiral separation of carvedilol in human plasma using UV-DAD detector.
- A total of 500  $\mu\text{l}$  of plasma spiked with carvedilol and subjected to protein precipitation. Afterward, 1 ml of supernatant diluted with alkaline water. Dispersive solvent and extraction solvent injected rapidly and the sedimented phase was collected and dried using  $\text{N}_2$  stream and residue dissolved in water + ACN (1:1) for analysis with CE.
- The separation performed on capillary column (50 cm  $\times$  50  $\mu\text{id}$ ) by adding CM- $\beta$ -CD to BGE as a chiral selector under stacking mode (15 kV  $\times$  30 s).
- US FDA guideline was used for validation of the developed method.

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