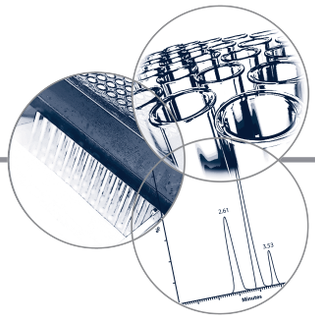

Analysis of losartan and carvedilol in urine and plasma samples using a dispersive liquid–liquid microextraction isocratic HPLC–UV method

Background: A simple, precise and sensitive HPLC method has been developed for simultaneous determination of carvedilol and losartan in human plasma and urine samples. The analytes were extracted by a dispersive liquid–liquid microextraction method. A mobile phase of 15 mM sodium dihydrogen phosphate buffer (pH 4.0)/acetonitrile/2-propanol (70/27.5/2.5, v/v/v) was used to separate the drugs using a Waters® ODS column (250 × 4.6 mm) and detected by a UV detector at 222 nm. **Results:** The developed method is selective for studied drugs possessing a linearity range of 0.1–1.0 and 0.05–0.75 µg/ml, respectively, for losartan and carvedilol with precision <15%. The accuracy is better than 15% and the mean recovery of carvedilol and losartan was 98.9 and 100.2% for plasma and 100.7 and 100.5% for urine samples, respectively. **Conclusion:** The developed method is applicable for therapeutic drug monitoring and PK analyses.

Hypertension, arrhythmia and high cholesterol level are symptoms of disorders of the heart and blood vessels called cardiovascular diseases (CVDs). According to a WHO report, Global Atlas on Cardiovascular Disease Prevention and Control, by 2030 approximately 23.6 million people will die from CVDs [1]. This report states that although a large proportion of CVDs are preventable, they continue to rise mainly because preventive measures are inadequate. Tobacco use, unhealthy diet, low physical activity and harmful use of alcohol increase the risk of CVDs. Living a healthier lifestyle will prevent most CVDs. Beyond this, medical therapies are an essential part of CVD control. Control and management of hypertension has impressive effects on the health status of patients suffering from CVDs. Medical therapies can be conducted using two different approaches; that is, monotherapy and combined therapy. The studies demonstrate that less than one third of the hypertensive patients achieved the desired blood pressure by using monotherapy [2]. The combination of two or more drugs is more efficient. The combination of thiazides, β-blockers, acetyl choline esterase inhibitors, calcium-channel blockers and angiotensin II receptor antagonists have been studied and the results reveal that this method leads to higher efficacy (approximately five-times) and a reduction of side effects due to lowering the dose of each drug [3]. It is well known that the efficacy of these drugs is associated with their body level, and their quantification in biologic samples is required in order to provide more efficient drug therapy. Quantification of the mentioned cardiovascular drug families is widely studied [4]. Review of the published papers showed that several analytical methods were developed for simultaneous determinations of β-blockers, calcium-channel blockers, diuretics and angiotensin II receptor antagonists, whereas the simultaneous analyses of losartan and carvedilol was rarely studied [4,5].

Carvedilol (Table 1) is a nonselective β-blocker used for treatment of heart failure and hypertension, usually in combination with other drugs (e.g., losartan). It works by relaxing blood vessels and reducing heart rate to improve blood flow and decrease blood pressure. Losartan (Table 1) is the first orally available angiotensin II receptor and is used as an antihypertensive agent. Losartan blocks the vasoconstrictor and aldosterone-secreting effects of angiotensin II by blocking AT1 receptors.

Carvedilol and losartan undergo first-pass metabolism and 2 and 4% of drugs are excreted unchanged in the urine. The therapeutic levels of these drugs are reported in Table 1. The combination of these drugs has advantages in heart failure and their cardiovascular preventive mechanisms have been studied [6,7].

HPLC method is the most commonly used bioanalytical technique, providing precise, reliable and reproducible results. It suffers from some limitations, such as the matrix effect,

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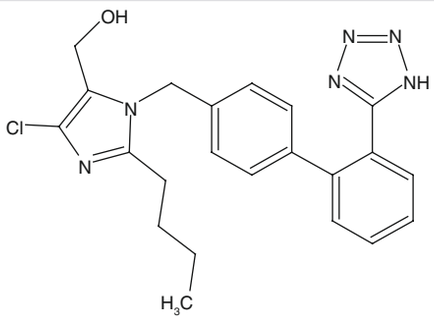
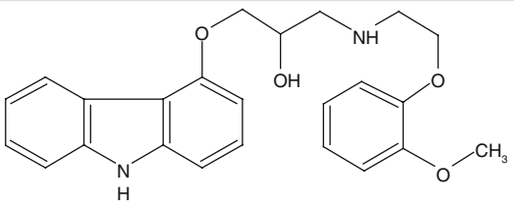
Table 1. Molecular shape, PK and physicochemical properties of losartan and carvedilol.

Name	Losartan	Carvedilol
Molecular structure		
Therapeutic range (μg/ml)	0.2–0.65	0.02–0.16
$logD^{a}_{pH=7.4}$	1.59	3.29
logP	4.01	4.19
pKa†	4.42	8.16

†*Physicochemical properties calculated using ACDLabs software.*

compromised selectivity and reduced sensitivity of the analyte in the pretreated biological matrices. These facts make sample treatment or extraction necessary for selective isolation of the analyte, minimization/elimination of matrix components and concentration of the analyte if necessary [8].

Conventional sample preparation methods (e.g., liquid–liquid extraction and SPE) have been applied for the determination of carvedilol and losartan. Newer microextraction techniques have been proposed for the separation and preconcentration of drugs from biological fluids. **Dispersive liquid–liquid microextraction** (DLLME) as a novel liquid microextraction technique with less solvent consumption was first reported by Rezaee *et al.* [9]. A ternary solvent extraction system, including dispersive solvent, extraction solvent and aqueous samples containing target analyte, provides a simple, rapid and cheap method that leads to a high enrichment factor and high recovery. DLLME has been widely applied for environmental and food samples [9–19], while its application for drug analysis in biological matrices has been rarely studied (Table 2) [20–47]. Urine was studied as the most common biological sample, and plasma, serum, whole blood and saliva were analyzed for the applicability of DLLME for drug extraction from biological samples.

DLLME involves two steps: the mixing of extracting and dispersing solvents into an aqueous sample to form an emulsion, which facilitates the extraction of the analyte to the extraction solvent; and the dispersion is removed by centrifugation and the extracting solvent containing analytes is used for the analysis.

In the present work, DLLME was employed for the simultaneous extraction of carvedilol and losartan in human plasma and urine. A simple isocratic HPLC–UV method was developed for the simultaneous analysis of extracted drugs and the developed method was validated according to the US FDA recommendations for bioanalytical method validation [48].

Key Term

Dispersive liquid–liquid microextraction: Method used for analyte extraction from biologic and nonbiologic samples.

Experimental

Chemicals

Carvedilol (European Pharmacopea) and losartan were kindly provided by Sobhan Pharmaceutical Co. (Rasht, Iran), sodium dihydrogen phosphate, phosphoric acid and sodium hydroxide purchased from Merck (Germany), and sodium chloride, HPLC grade acetonitrile, 2-propanol, chloroform, acetone, tetrachloromethane, dichloromethane, 1,1 dichloroethane and methanol were purchased from Scharlau Chemie (Barcelona, Spain). Double distilled water (prepared daily in the laboratory) was used in the study.

Apparatus

The Knauer® (Berlin, Germany) chromatographic system equipped with a WellChrom Maxi-Star K-1000 pressure pump, an online Biotech 2003 multichannel degasser, a WellChrom K-2500 spectrophotometer, a data processor using EuroChrom 2000 software, Waters® precolumn cartridge (Milford, PA, USA) followed by a C18 ODS-3 (5 μm) Waters analytical column (250 × 4.6 mm) incubated in

Table 2. Applications of dispersive liquid–liquid microextraction for extraction of drugs and drug-like chemicals from biological samples.

Analyte	Dispersant	Extractant	Sample	Pretreatment	Validation	Analysis method	Ref.
Clozapine and chlorpromazine	Ethanol	Carbon tetrachloride	Human urine	Centrifugation after freezing, with sediment after centrifugation, dissolved by methanol and filtered before injection	Yes	HPLC–UV	[20]
Clenbuterol	Methyl tert-butyl ether	Chloroform	Swine urine	No treatment	No validation	HPLC–UV	[21]
Imipramine, amitriptyline, clomipramine, desipramine and nortriptyline	Methanol	Carbon tetrachloride	Human urine	Urine and acetonitrile and centrifugation dilution of supernatant with water	No stability, selectivity and sensitivity	LC–MS	[22]
Cannabidiol, D9-tetrahydrocannabinol and cannabinol	Tetradecyl trimethyl ammonium bromide	Toluene	Human urine	No treatment	No validation	HPLC–UV	[23]
7-aminoflunitrazepam	Isopropyl alcohol	Dichloromethane	Human urine	Urine and ammonia and centrifugation and using supernatant for DLLME	No stability, selectivity and sensitivity tests	LC–ESI-MS/MS	[24]
Amitryptiline, clomipramine and thioridazine	Acetonitrile	Carbon tetrachloride	Human urine	Freezing, centrifugation, filtration with sediment after DLLME, centrifugation, supernatant dissolved by acetonirile and filtered before injection	Yes	HPLC–UV	[25]
Morphine, codeine and thebaine	Acetone	Chloroform	Human urine	Freezing, centrifugation, filtration, dilution of supernatant and DLLME	No stability, selectivity and sensitivity tests	HPLC–UV	[26]
Tetrahydropalmatine and tetrahydroberberine	Methanol	Chloroform	Rat urine	Freezing, centrifugation, filtration with sediment after DLLME, water addition, centrifugation and supernatant discarding	No stability	HPLC–UV	[27]
Guaifenesin enantiomers	THF	Dichloromethane	Human urine	Dilution	No stability	HPLC–fluorescence	[28]
Celastrol	Methanol	[C6MIM][PF6]	Human urine	Dilution	No stability	HPLC–UV	[29]
Emodin and its metabolites	Acetonitrile	[C6MIM][PF6] and THF	Human urine	Dilution	No stability	LC–MS/MS and HPLC–UV	[30]

CCT: Cold column trapping; CTAB: Cetyltrimethylammonium bromide; DAD: Diode array detector; DLLME: Dispersive liquid–liquid microextraction; MD: Multidimensional; THF: Tetrahydrofuran.

Table 2. Applications of dispersive liquid–liquid microextraction for extraction of drugs and drug-like chemicals from biological samples (cont.).

Analyte	Dispersant	Extractant	Sample	Pretreatment	Validation	Analysis method	Ref.
Four synthesized metabolites of mequindox	Methanol	1,1,2,2-tetra chloroethane	Human urine	Freezing, centrifugation, filtration with sediment after DLLME, centrifugation, supernatant dissolved by acetonitrile and filtered before injection	No stability	HPLC–UV	[31]
Alprazolam, oxazepam and diazepam	Ethanol	Dichloromethane	Human urine	Dilution	No stability, selectivity and sensitivity tests	HPLC–UV	[32]
Irbesartan and valsartan	Acetone	[C8MIM][PF6]	Human urine	Urine diluted with methanol, frozen for 10 h, centrifuged and filtered	No stability	HPLC–DAD	[33]
Methamphetamine and ecstasy	Methanol	Butylchloroformate	Human urine	The method coupled with molecularly imprinted polymer	No stability	GC–MS	[34]
Bisphenol A and bisphenol B	Acetonitrile	Tetrachloroethylene	Human urine	No treatment	No stability	MDGC/MS	[35]
Amphetamine and methamphetamine	Sodium dodecyl sulfate solution	1-undecanol	Human urine	Dilution	No stability, selectivity and sensitivity tests	HPLC–UV	[36]
Indomethacin, ketoprofen,	Methanol	[BMIM][PF6]	Human urine	Filtration and dilution	No stability, selectivity and sensitivity tests	HPLC–UV	[37]
Rifaximin	Methanol	1-butyl-3-methylimidazolium hexafluorophosphate	Rat serum	No treatment	No validation	HPLC–DAD	[38]
Curcumin	Acetone	1-dodecanol	Serum	Precipitation with acetone followed by CCT-DLLME	No validation	HPLC–UV	[39]
Carbamazepine	Ethanol	Chloroform	Human urine and plasma	Urine, dilution plasma, precipitation with acetonitrile	No validation	HPLC–UV	[40]
Methadone	Methanol	Chloroform	Human urine, plasma, saliva and sweat	All samples frozen, centrifuged and supernatant was diluted	No stability, selectivity and sensitivity tests	HPLC–UV	[41]
Fentanyl, alfentanil and sufentanil	Methanol	Chloroform	Human urine and plasma	Urine: filtration and dilution plasma: filtration, dilution and precipitation with methanol	No stability, selectivity and sensitivity tests	HPLC–UV	[42]

CCT: Cold column trapping; CTAB: Cetyltrimethylammonium bromide; DAD: Diode array detector; DLLME: Dispersive liquid–liquid microextraction; MD: Multidimensional; THF: Tetrahydrofuran.

Table 2. Applications of dispersive liquid–liquid microextraction for extraction of drugs and drug-like chemicals from biological samples (cont.).

Analyte	Dispersant	Extractant	Sample	Pretreatment	Validation	Analysis method	Ref.
Sulfamethazine, sulfamethoxypyridazine, sulfamethoxydiazine, sulfamethoxazole, sulfadimethoxine and sulfaphenazole	Methanol	Ionic liquid	Pig plasma	Precipitation with methanol, dryness of supernatant, dilution with acetate buffer	No stability, selectivity and sensitivity tests	HPLC–fluorescence	[43]
Letrozole	Acetone	Chloroform	Human urine and plasma	Urine: dilution Plasma: precipitation with acetonitrile, filtration and dilution	No validation	HPLC–UV	[44]
Warfarin	Methanol	1-octanol	Plasma	Precipitation with trichloroacetic acid, filtration and dilution	No stability, selectivity and sensitivity tests	HPLC–UV	[45]
Ketoconazole and econazole nitrate	CTAB	Chloroform	Blood		No stability, selectivity and sensitivity tests	HPLC–UV	[46]
Volatile aldehyde biomarkers	Methanol	1-dodecanol	Blood	Precipitation with methanol and dilution	No stability, selectivity and sensitivity tests	HPLC–UV	[47]

CCT: Cold column trapping; CTAB: Cetyltrimethylammonium bromide; DAD: Diode array detector; DLLME: Dispersive liquid–liquid microextraction; MD: Multidimensional; THF: Tetrahydrofuran.

a space column oven (Grace Vydac Inc.; Worms, Germany). The solvents were filtered through a 0.45 µm membrane filter (Millipore Corp., MA, USA) using a Millipore vacuum pump. The samples were vortexed using a Labtron® shaker model LS-100 (Mumbai, India) and centrifuged by a Sanyo® Micro-centaur (MSBO10.CX2.5) centrifuge (UK) and a Shimifan (CE.148) centrifuge (Tehran, Iran). A Metrohm® (744) pH meter (Herisau, Switzerland) equipped with a glass electrode (an AgCl reference system and KCl 3 M as electrolyte), was used to measure pH of solutions. A Liarre Strasonic 18–35 ultrasonic bath (Bologna, Italy) was used to degas the mobile phase prior to use. A 100-µl Hamilton® syringe (TN, USA) was used to transfer the sample and injection to the HPLC system.

■ Standard solutions & biologic samples

A total of 25 mg of each drug was weighted and dissolved in 25 ml methanol to prepare (1000 µg/ml) stock solutions, and was stored at 4°C. The working standard solutions were prepared daily by dilution of stock solutions using the mobile phase. Combined working standards containing losartan and carvedilol were prepared similar to working standards.

Drug-free plasma samples (donated by Blood Transfusion Organization of Eastern Azerbaijan, Tabriz, Iran) stored in polypropylene microtubes and frozen at -20°C. Drug-free urine samples were collected from a healthy male volunteer who did not take any medicine within 2 months.

Spiked plasma (1 µg/ml) and urine (10 µg/ml) samples were prepared daily by the dilution of the stock solution by drug-free plasma and urine samples. This dilution method was used to do the optimization procedure using the samples, which are almost unique to the clinical plasma and urine samples. After vortexing the spiked plasma and urine for 20 s they were equilibrated for 10 min before each analysis. Calibration standards were prepared (0.1–1.0 µg/ml) and (0.05–0.75 µg/ml) for plasma and (0.05–1.0 µg/ml) and (0.02–1.0 µg/ml) for urine samples, respectively, for losartan and carvedilol using a similar method.

Stability assays were conducted using the QC samples, which were prepared by the low, middle and high concentrations of each analyte spiked in urine and plasma.

■ Extraction procedure

To a 15-ml conical glass tube, 10 ml of spiked urine sample was added followed by the addition

of the 1-ml sodium dihydrogen phosphate buffer solution. Then the mixture of the dispersive/extraction solution (400/160 and 500/100 µl, respectively, for the urine and plasma samples) was quickly and vigorously injected to the tube using a 1.0 ml syringe (Hamilton; TN, USA). A cloudy solution quickly formed due to dispersion of extraction solvent in aqueous sample greatly enlarging the contact area between the extraction solvent and aqueous phase.

The emulsion was centrifuged (2500 rpm for 5 min) and 50 µl of the extraction solvent that was collected at the bottom of the tube was transferred to a microtube after discarding the supernatant. After evaporation of the solvent using a 60°C oven, the residue was dissolved using 50 µl of the mobile phase and injected into the HPLC system.

In the case of plasma samples, the experiments demonstrated that a primary protein-precipitation step was necessary in which 1 ml acetone was added to 1 ml plasma sample and 1 ml of the supernatant was transferred to the conical tube followed by the addition of 0.5 ml buffer solution and 8.5 ml NaCl (4.7%) solution. Acetone was used for a precipitation procedure to avoid interference of another solvent with the extraction procedure. It should be noted that acetone was also used as a disperser solvent and any residual acetone in the supernatant cannot cause any problems, which is not the case for other precipitating solvents. The remaining (the addition of dispersive/extraction solution mixture, centrifugation, solvent evaporation and resolvation) was similar to the urine samples.

Extraction optimization

In order to reach the optimized conditions of extraction the effective parameters (i.e., ionic strength or salt effect, type and concentration of the extraction solvent, type and concentration of the dispersive solvent, pH and concentration of the buffering agent, and the rate and duration of the centrifugation) were studied using a systematic, 'one-factor-at-a-time' experimental design. To do this, the study range of each parameter (Table 3) was selected according to the theoretical considerations (the structural features and physicochemical properties of the losartan and carvedilol). Table 1 shows the molecular structures along with the physicochemical and PK properties of the investigated drugs.

HPLC–UV optimization

The previously developed method was re-optimized (according to the flow rate, temperature, the buffer concentration and pH, organic solvent concentration and the equilibration time) and used for the separation of carvedilol and losartan [4]. The absorption wavelengths were optimized to reach a single wavelength, which possesses the best sensitivity and peak shape, and the resulting condition was used to analyze the biological samples.

Method validation

The developed method was validated according to the FDA recommendations for bioanalytical method validations. The calibration, linearity, LOD, LOQ, LLOQ and ULOQ, intra- and inter-day precisions, accuracy, recovery, selectivity, specificity and stability (room temperature, refrigerator [4°C and freeze–thaw]) and robustness were tested.

The mean of three calibration curves (produced on three different days) was used for the validation studies. The calibration range was selected, which included the therapeutic concentration of the drugs, and was considered as the linear range. The mean of three replications

Table 3. Details of the optimized parameters.

Optimized parameter	Plasma	Urine
pH	1–8	1–8
Buffer concentration (mM)	10–275	6–14
Dispersive solvent type	Acetone, methanol, tetrahydrofuran and acetonitrile	Acetone, methanol, tetrahydrofuran and acetonitrile
Dispersive solvent concentration (ml/10 ml)	0.2–0.7	0.2–0.7
Extraction solvent type	$CHCl_3$, CCl_4, CH_2Cl_2 and $C_2H_4Cl_2$	$CHCl_3$, CCl_4, CH_2Cl_2 and $C_2H_4Cl_2$
Extraction solvent concentration (ml/10 ml)	0.08–0.12	0.14–0.19
NaCl concentration (g/ml)	0.01–0.07	0.01–0.07
Centrifuge rate (rpm)	1250–5000	1250–5000
Centrifuge duration (min)	2.5–10	2.5–10
Sample dilution	4–10-times	No dilution needed

was used for all quantifications. The lowest concentration of calibration curve was selected as the LLOQ while the %CV of three replications was less than 20%, and for ULOQ the highest concentration of calibration curve was selected while the %CV of three replicates was less than 15%. The LOD and LOQ values were calculated for each drug using the following equations:

$$LOD = 3.3^{*}\left(\frac{S_b}{a}\right)$$

EQUATION 1

$$LOQ = 10^{*}\left(\frac{S_b}{a}\right)$$

EQUATION 2

where s_b is the standard error of intercept and a is the slope of the calibration curve.

Five replicates of low, medium and high concentrations of drugs in five different days were used for inter- and intra-day precisions studies. By comparing the obtained concentrations of five different plasma samples with nominal values the accuracies were checked by computing the relative errors (%) using:

$$\%RE = 100 \times \left(\frac{\text{Calculated conc. - nominal conc.}}{\text{Nominal conc.}}\right)$$

EQUATION 3

The recovery of the sample preparation method was calculated using the following equation for five biological samples:

$$\text{Recovery (\%)} = \frac{100 \times \text{measured conc.}}{\text{Nominal conc.}}$$

EQUATION 4

The freeze–thaw stability was assessed after three freeze–thaw cycles at 12-h intervals. The room temperature stability was assessed after 12 h of samples remaining at room temperature.

The robustness of the method for chromatographic separations was checked based on the effective parameters (pH, column temperature, organic modifier concentration and flow rate). Each parameter was varied in three levels and the effects on the retention times and peak areas were studied. The robustness of the sample preparation and extraction procedures was validated by slight variation of the concentration of dispersive and extraction solvent, pH and concentration of the buffering agent, and the rate and duration of the centrifugation. In addition, the effect of the temperature variations during a day on the extraction procedure was validated by preparation and extraction of the samples at different times of the day (i.e., morning, noon and afternoon).

Results & discussion

■ Optimized extraction conditions

To find the optimal conditions of the studied parameters according to the 'one factor' experimental design, 44 experiments were designed separately for plasma and urine samples and the results are shown in FIGURE 1. In all graphs the extraction efficiency (peak area) was plotted against the desired parameter.

■ Buffer pH optimization

The pH 2.0 was selected for the buffer (10 mM phosphate buffer) according to FIGURE 1A for both urine and plasma samples. The extraction efficiency in acidic pH is higher than the basic solutions for both drugs. We avoided more acidic pHs in order to avoid possible damage of the lower pHs to the HPLC column.

■ Selection of extraction solvent type & volume

Some heavy organic solvents (CCl_4, $CHCl_3$, CH_2Cl_2 and CH_3Cl) with relative density values above 1 g/ml, were selected according to the properties needed to extract the analytes efficiently (i.e., higher density than water, good chromatographic behavior or ease of evaporation, extraction capability of interested compound, low solubility in water). FIGURE 1B shows the extraction efficiencies of each solvent for urine and plasma samples. Chloroform showed higher efficiency and was selected as extraction solvent. To check the extraction solvent volume on the extraction efficiency, six different volumes of chloroform in a given volume (500 µl) of acetone were used. The optimized volumes (100 and 160 µl for plasma and urine, respectively) were selected according to FIGURE 1C. Our experiments showed that different volumes of extraction solvent are needed for different biological matrices and this finding shows the necessity of optimization using biological samples instead of water samples when our aim is to use DLLME for drug analysis in biological samples. Similar trends were observed for extraction solvent type and volume for the studies of drugs.

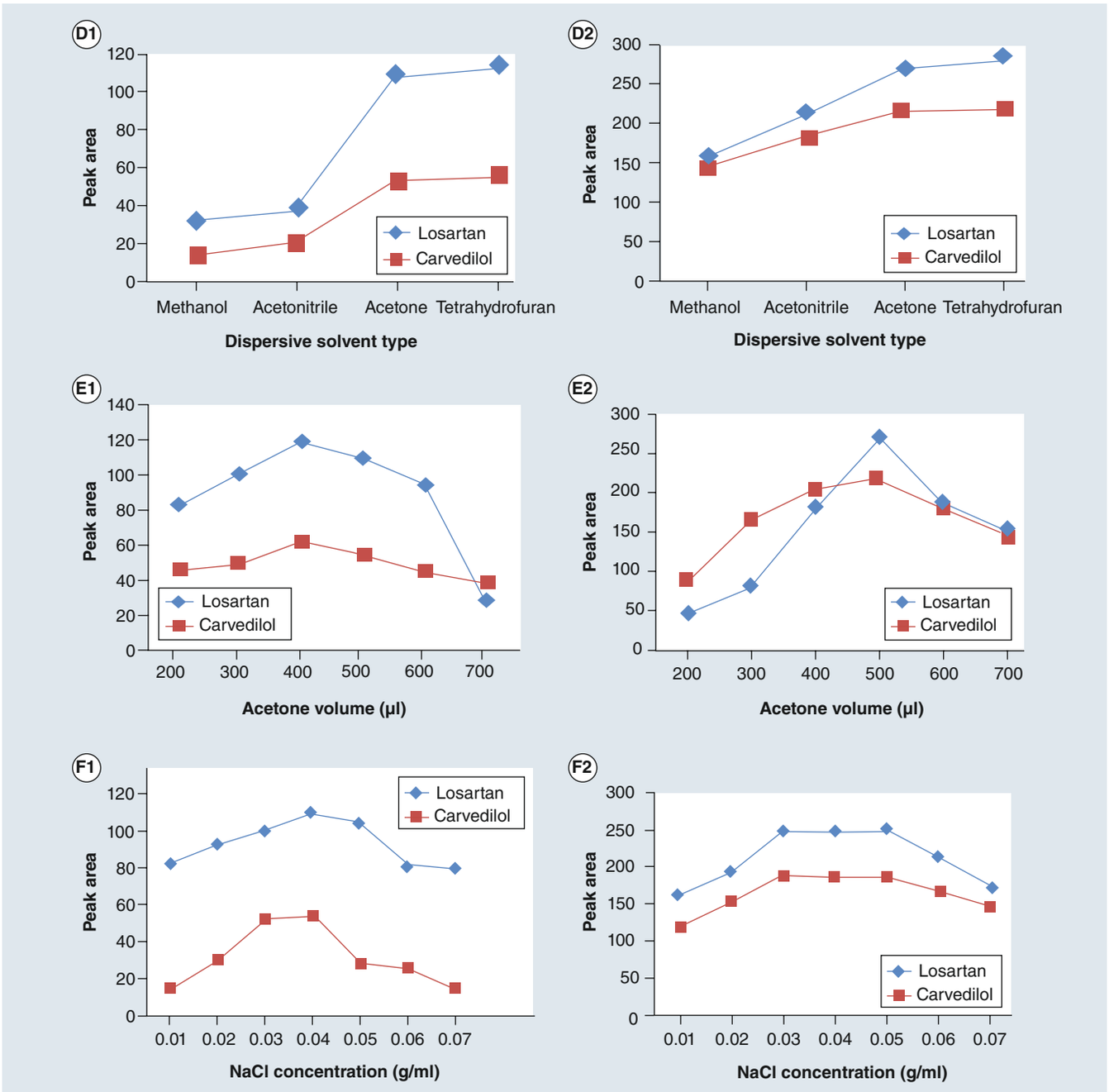


Figure 1. One factor at a time experimental design for the optimization of extraction procedure (cont.). Optimization of **(D)** dispersive type, **(E)** volume and **(F)** salt concentration in **(1)** urine and **(2)** plasma samples.

■ Selection of dispersive solvent type & volume

Common dispersive solvents (i.e., methanol, acetonitrile, acetone and tetrahydrofuran) were studied to determine the most suitable dispersive solvent. As seen in Figure 1D, acetone and tetrahydrofuran were able to disperse the chloroform better than acetonitrile and methanol in both urine and plasma samples. Acetone was selected as the dispersive solvent because of its lower toxicity in comparison with tetrahydrofuran and because it is present in the samples as a protein precipitation agent. The optimization of acetone volume (Figure 1E) was carried out in the presence of the optimized volume of chloroform and optimized volumes of 400 and 500 µl were obtained for urine and plasma samples, respectively. The effect of

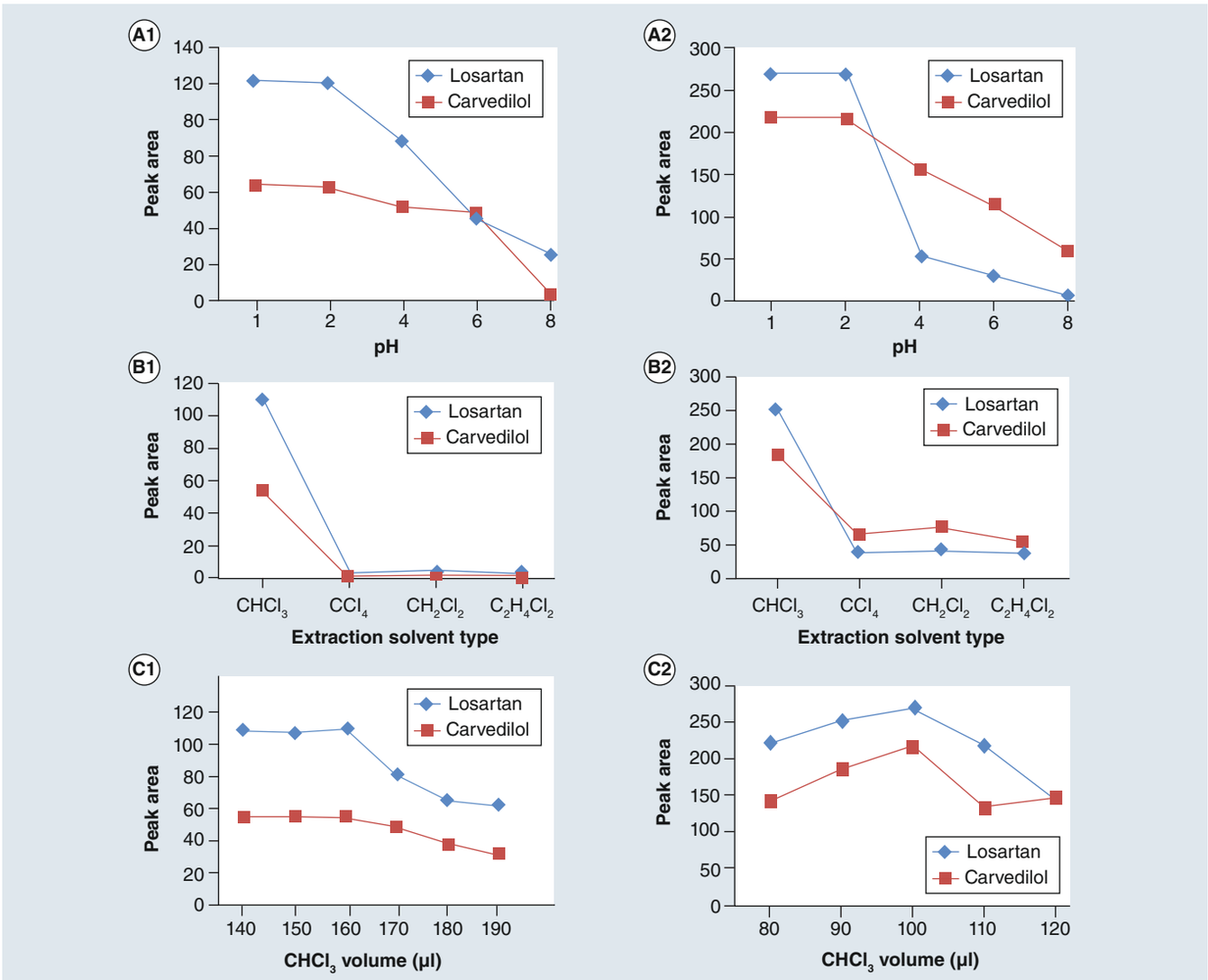


Figure 1. One factor at a time experimental design for the optimization of extraction procedure. Optimization of **(A)** pH, **(B)** extraction type and **(C)** volume in **(1)** urine and **(2)** plasma samples.

individual factors on the extraction efficiency showed similar trends on both drugs studied.

■ Effect of salt addition

To check the possibility of the 'salting-out effect' during extraction, the effect of NaCl concentration (ranging from 0.01 to 0.07 g/ml) additions on extraction efficiency was studied. As shown in Figure 1F, the salt addition improved the extraction of the drugs both in urine and plasma samples and the total concentration of 0.04 g/ml was selected as optimized value.

■ Optimization of buffer concentration

Figure 1G shows the optimization of the effect of the buffering agent concentration on the extraction. The experiment showed that the optimized condition was achieved for urine samples in lower concentration (i.e., 10 mM) in comparison with plasma samples (i.e., 250 mM). pH adjusting without buffering agents significantly reduced the efficiency.

■ Effect of centrifugation rate & duration

The centrifugation rate and duration were validated using optimized values for the parameters studied (Figures 1H & 1I) and 2500 rpm and 5 min were selected as the optimized rate and duration.

■ Biological matrix effect on extraction

Urine samples can be used for DLLME without any pretreatment under the optimized conditions. Using acidic pH that adjusted

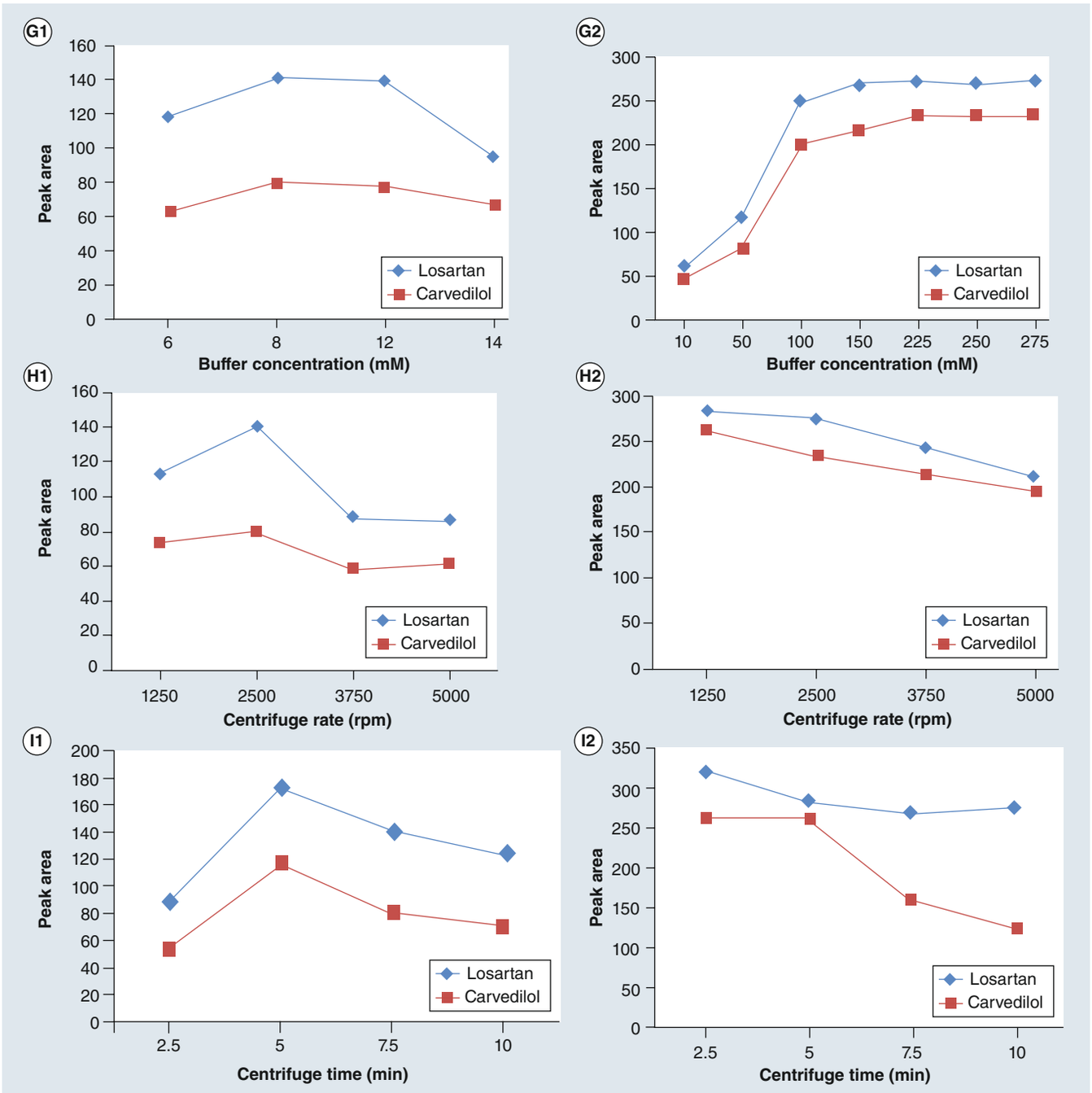


Figure 1. One factor at a time experimental design for the optimization of extraction procedure (cont.). Optimization of **(G)** buffer concentration **(H)** centrifugation rate and **(I)** time in **(1)** urine and **(2)** plasma samples.

with buffer solution, no white sediments were observed in our experiments. These sediments were mentioned in earlier studies [20–22,24–37]. With reference to these findings no pretreatment is needed for urine samples.

For plasma samples, as mentioned in previous studies, protein precipitation is required [39–45]. Omitting this step results in white sedimentation, which reduced the volume of the extraction solvent and, consequently, the extraction efficiency. In addition, an extra step of dilution is needed for plasma samples that were optimized (Figure 2) and the results showed that the five-times dilution (i.e., 8.5 ml of aqueous NaCl 4.7% solution and 0.5 ml buffer solution to receive total concentration of

4%) is enough. Our findings demonstrated that dilution without precipitation or precipitation without dilution is not sufficient for a suitable extraction.

■ Chromatographic condition

The mobile phase of 15 mM sodium dihydrogen phosphate buffer (pH 4.0)/acetonitrile/2-propanol (70/27.5/2.5, v/v/v) was used for the separation of losartan and carvedilol in 10 min. The flow rate was 2 ml/min and the column temperature was 25°C. FIGURE 3A–D shows the chromatograms of drug-free plasma, drug-free urine, spiked plasma and spiked urine samples, respectively (0.5 µg/ml).

Validation report

■ Linearity & calibration curves

By plotting the response function (peak area) against the corresponding concentrations, the calibration curves were obtained. Five increasing levels for losartan (0.1–1.0 µg/ml), and carvedilol (0.05–1.0 µg/ml) were analyzed on three different days to construct the corresponding calibration curves. Correlation coefficients ($r > 0.99$ for all calibrations) were used for evaluation of linearity. TABLE 4 shows the details (slope, intercept, standard errors and

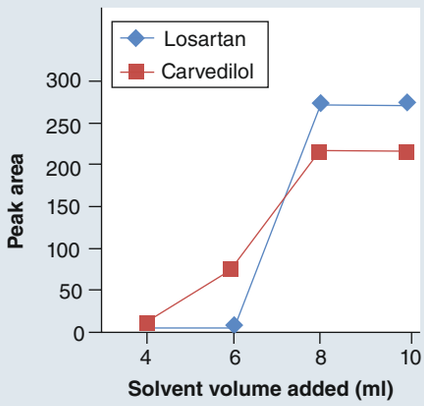


Figure 2. The effect of dilution of plasma samples on extraction.

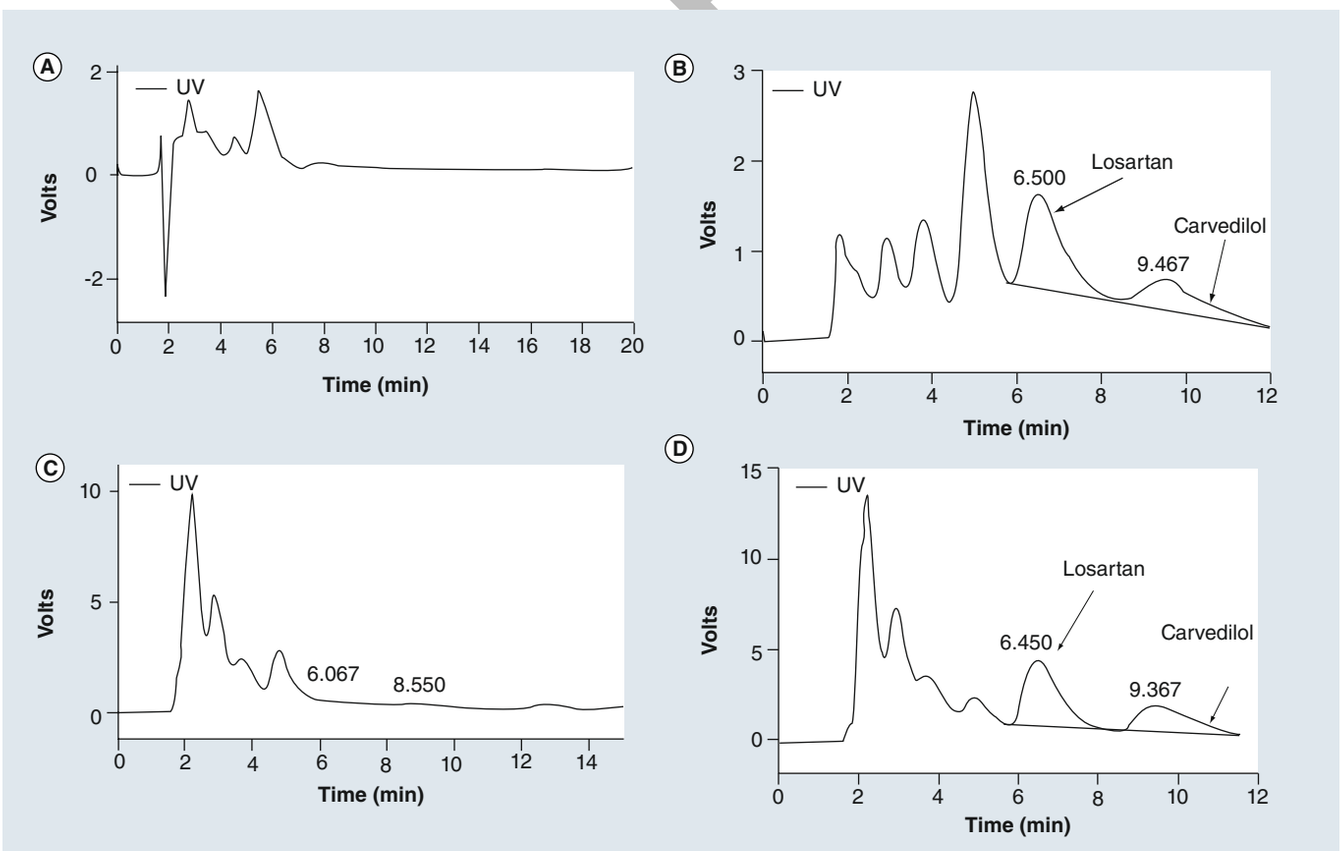


Figure 3. Chromatograms of free drug and spiked urine and plasma samples. The chromatograms of the free drug and spiked (0.5 µg/ml) in **(A & C)** plasma and **(B & D)** urine samples (mobile phase of 15 mM sodium dihydrogen phosphate buffer (pH 4.0)/acetonitrile/2-propanol [70/27.5/2.5, v/v/v]).

Table 4. Validation data of the proposed method for quantification of losartan and carvedilol in human plasma and urine.

Parameters	Plasma		Urine	
	Carvedilol	Losartan	Carvedilol	Losatran
Linear range (µg/ml)	0.05–0.75	0.1–1.0	0.02–1.0	0.05–1.0
Slope	0.429	0.618	0.229	0.322
Slope standard errors	0.007	0.015	0.004	0.003
Intercept	44.16	-10.55	3.443	14.52
Intercept standard errors	2.385	3.188	0.142	1.262
Correlation coefficient	0.998	0.996	0.999	0.999
Number of data points	5	5	7	6
LOD (µg/ml)	0.014	0.011	0.004	0.007
LOQ (µg/ml)	0.047	0.036	0.014	0.023
LLOQ (µg/ml)	0.05	0.1	0.02	0.05

Table 5. Intra-assay precision and accuracy of calibration standards.

Sample	Drug name	Nominal concentration (µg/ml; n = 3)	Found concentration (µg/ml; n = 3)	Precision (%CV)	Accuracy (%RE)
Plasma	Losartan	0.100	0.095	10.7	-5.0
		0.200	0.186	7.9	-7.0
		0.500	0.510	9.6	2.022
		0.750	0.761	7.0	1.5
		1.000	0.981	3.7	-1.9
	Carvedilol	0.050	0.046	9.4	-8.0
		0.100	0.106	11.3	6.0
		0.200	0.208	11.0	4.0
		0.500	0.507	9.5	1.4
		0.750	0.743	8.8	-0.9
Urine	Losartan	0.050	0.047	11.6	-6.0
		0.100	0.104	10.9	4.0
		0.200	0.209	10.0	4.5
		0.500	0.506	3.1	1.2
		0.750	0.732	3.0	-2.4
	Carvedilol	0.050	0.047	5.4	-6.0
		0.100	0.107	4.2	7.0
		0.200	0.214	6.6	7.0
		0.500	0.495	6.8	-1.0
		0.750	0.740	2.6	-1.3

r values) of mean calibrations and corresponding validation parameters (LOD, LLOQ, ULOQ) along with therapeutic levels of the studied drugs. The %CV and relative errors percentage values for calibration data are reported in Table 5.

■ Selectivity & specificity

Six drug-free human plasma and urine samples were selected randomly and analyzed in order to check the specificity of the developed method, and the results demonstrated that there are no plasma- or urine-contributing peaks for losartan and carvedilol. In order to check the selectivity, some other cardiovascular drugs (i.e., propranolol, atorvastatin, amiodaron, diltiazem, hydrochlorothiazide, amiloride, amlodipine, lovastatin, simvastatin, lisinopril, captopril, enalapril, atenolol and nifedipine) and some analytes such as caffeine, ibuprofen, acetaminophen, diazepam and salicylic acid, were analyzed. The experiments demonstrated that, although some of them eluted with the developed mobile phase (i.e., salicylic acid

Table 6. Assay precision and accuracy of QC samples.

Sample	Drug name	Concentration (µg/ml)	Intra-assay precision (%CV)	Inter-assay precision (%CV)	Accuracy (%RE)
Plasma	Losartan	0.200	7.0	9.1	-6.1
		0.500	5.4	8.8	-7.6
		0.750	7.6	8.1	6.8
	Carvedilol	0.200	7.5	8.8	4.4
		0.500	5.7	10.8	4.7
		0.750	7.7	10.3	-3.6
Urine	Losartan	0.200	8.2	9.0	4.1
		0.500	8.8	9.9	3.0
		0.750	8.6.	8.9	0.5
	Carvedilol	0.200	7.9	8.2	2.0
		0.500	8.1	9.2	2.9
		0.750	6.0	9.6	-3.7

Table 7. Absolute and mean recoveries for losartan and carvedilol in plasma and urine samples.

Sample	Drug name	Nominal concentration (µg/ml)	Determined concentration (µg/ml; n = 5)	Recovery (%)	Mean recovery (%)	Precision of recovery (%RE)
Plasma	Losartan	0.100	0.096	95.9	98.9	-4.1
		0.200	0.189	94.5		-5.5
		0.500	0.521	104.2		4.2
		0.750	0.767	102.2		2.2
		1.000	0.975	97.5		-2.5
	Carvedilol	0.050	0.047	95.6	100.2	-4.4
		0.100	0.104	103.9		3.9
		0.200	0.204	101.9		1.9
		0.500	0.495	98.9		-1.1
		0.750	0.760	101.4		1.4
Urine	Losartan	0.050	0.048	96.1	100.7	3.9
		0.100	0.105	105.2		5.2
		0.200	0.207	103.8		3.8
		0.500	0.505	101.1		1.1
		0.750	0.729	97.3		-2.7
	Carvedilol	0.050	0.047	94.9	100.5	-5.1
		0.100	0.104	103.8		3.8
		0.200	0.211	105.8		5.8
		0.500	0.468	98.7		-1.3
		0.750	0.743	99.1		-0.9

diltiazem, propranolol and diazepam), they do not interfere with the studied drugs.

Precision & accuracy

Table 6 shows the results of intra-assay precision and accuracy of calibration standards. All %CV values for LLOQ and higher concentrations were below 15%. Inter- and intra-assay precision along with accuracy for QC samples are listed in Table 7. The results of these validation experiments demonstrated that the developed method is both accurate and precise.

Recovery

The recoveries are summarized in Table 8. The mean recoveries for losartan and carvedilol were 98.9 and 100.2% in urine, and 100.7 and 100.5% in plasma samples, respectively.

Stability

The stability experiments proved that the samples are stable both in plasma and urine samples after 12 h at room (25.0 ± 2.0°C) and refrigerator (4.0°C) temperatures. The freeze–thaw cycles caused no significant instability (Table 9).

Table 8. Stability study of plasma samples†.

Drug	Concentration (µg/ml)	Freeze–thaw stability		Room temperature stability		4°C	
		Concentration found (µg/ml)	Accuracy (%RE)	Concentration found (µg/ml)	Accuracy (%RE)	Concentration found (µg/ml)	Accuracy (%RE)
Losartan	0.200	0.176	-12.0	0.195	-2.3	0.182	-6.2
	0.500	0.525	5.2	0.528	5.6	0.516	3.3
	0.750	0.715	-4.7	0.770	2.6	0.738	-1.6
Carvedilol	0.200	0.187	-6.3	0.213	6.6	0.196	-1.7
	0.500	0.465	-6.8	0.470	-6.1	0.486	-2.9
	0.750	0.663	-11.6	0.712	-5.1	0.726	-3.2

†*Freeze–thaw three times; room temperature 12 h; and 4°C stability data for studied drugs in plasma.*

Key Term

Therapeutic drug monitoring: Application of obtained information from drug bioanalysis in the management of a patient's drug therapy.

■ Robustness

By slight variation on the chromatographic parameters (pH 3.9, 4.0, 4.1), mobile phase of 15 mM sodium dihydrogen phosphate buffer (pH 4.0)/acetonitrile/2-propanol (70/27.5/2.5, v/v/v), (70.5/27/2.5 v/v/v), (69.5/28/2.5 v/v/v), (69.8/27.5/2.7 v/v/v), (70.2/27.5/2.3 v/v/v) and three replicated injections of 0.5 µg/ml spiked plasma and samples, the robustness of the developed method was validated. Negligible differences in peak area and retention times revealed the reliable robustness of the method. There is a considerable effect for 2-propanol variation that can be avoided by preparing high volumes (e.g., 500 ml) of the mobile phase.

The sample preparation and extraction was carried out at room temperature (25.0 ± 2.0°C) and the temperature variation of 2.0°C during the day has no significant effect on the extraction procedure. Concentration of the chloroform and acetone was varied by ±2.0% and the resulted peak areas showed no significant change in comparison with previous experiments. The pH of the buffer was slightly varied by 0.2 of a pH unit and the results revealed that the method is robust in a wide range of acidities. The variation of the concentration of the sodium dihydrogen phosphate had no effect on the extraction procedure. The method was slightly sensitive to the centrifugation time and rate and, if rigorously changed, would affect the method efficiency.

Conclusion

A rapid DLLME-HPLC–UV method has been developed to separate and quantify losartan and carvedilol from human plasma and urine samples. The developed method is applicable to PK, PD and **therapeutic drug monitoring** studies. The main advantages of the developed method are its ability to simultaneously extract and separate losartan and carvedilol. The developed method can be used as a precise analytical method in drug analysis laboratories.

Future perspective

Application of the developed method in the bioanalytical laboratories will enable scientists to precisely analyze low quantities of drugs in biological matrices without the need for high quantity of toxic solvents and materials.

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Table 9. Stability study of urine samples†.

Drug	Concentration (µg/ml)	Freeze–thaw stability		Room temperature stability		4°C	
		Concentration found (µg/ml)	Accuracy (%RE)	Concentration found (µg/ml)	Accuracy (%RE)	Concentration found (µg/ml)	Accuracy (%RE)
Losartan	0.200	0.208	4.1	0.215	7.7	0.212	6.1
	0.500	0.468	-6.4	0.493	-1.2	0.477	-4.4
	0.750	0.718	-4.2	0.725	-3.2	0.716	-4.4
Carvedilol	0.200	0.192	-3.9	0.202	1.3	0.209	4.8
	0.500	0.481	-3.7	0.481	-3.8	0.507	1.4
	0.750	0.707	-5.7	0.736	-1.7	0.712	-5.1

†*Freeze–thaw three times; room temperature 12 h; and 4°C stability data for studied drugs in urine.*

Executive summary

Background

- A dispersive liquid–liquid microextraction method was developed for the simultaneous extraction of the cardiovascular drugs (losartan and carvedilol) from urine and plasma samples.

Experimental

- An isocratic HPLC method was developed for the simultaneous analysis of losartan and carvedilol in extractions of urine and plasma samples.
- UV detector was used for the detection of drugs.
- The US FDA guideline has been used for the validation of the developed method.

Results

- The experiments resulted in a simple dispersive liquid–liquid microextraction method for simultaneous extraction of losartan and carvedilol following by an isocratic HPLC–UV method for their simultaneous analysis.

Discussion

- The developed method is applicable for the analysis of cardiovascular drugs in biologic samples.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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.

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