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Determination of five antiarrhythmic drugs in human plasma by dispersive liquid–liquid microextraction and high-performance liquid chromatography

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ABSTRACT

A fast and sensitive high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection was developed and validated for the simultaneous quantitation of five antiarrhythmic drugs (metoprolol, propranolol, carvedilol, diltiazem, and verapamil) in human plasma samples. It involves dispersive liquid–liquid microextraction (DLLME) of the desired drugs from 660 μL plasma and separation using isocratic elution with UV detection at 200 nm. The complete separation of all analytes was achieved within 7 min. Acetonitrile (as disperser solvent) resulting from the protein precipitation procedure was mixed with 100 μL dichloromethane (as an extraction solvent) and rapidly injected into 5 mL aqueous solution (pH 11.5) containing 1% (w/v), NaCl. After centrifugation, the sedimented phase containing enriched analytes was collected and evaporated to dryness. The residue was re-dissolved in 50 μL de-ionized water (acidified to pH 3) and injected into the HPLC system for analysis. Under the optimal conditions, the enrichment factors and extraction recoveries ranged between 4.4–10.8 and 33–82%, respectively. The suggested method was linear ($r^2 \geq 0.997$) over a dynamic range of 0.02–0.80 μg mL^{-1} in plasma. The intra- and inter-days relative standard deviation (RSD%) and relative error (RE%) values of the method were below 20%, which shows good precision and accuracy. Finally, this method was applied to the analysis of real plasma samples obtained from the patients treated with these drugs.

1. Introduction

Cardiovascular diseases (CVDs) are common causes of morbidity and mortality in all countries of the world and are caused by risk factors such as high blood pressure, coronary thrombosis, strokes, and renal failure [1]. Pharmacological treatment can reduce the risk of CVDs. Cardiovascular medications comprise the largest number of drugs that are used in controlling heart diseases [2]. Beta blockers (BBs) and calcium channel blockers (CCBs) are clinically important drugs and are used to treat a multitude of CVDs such as hypertension, angina pectoris, and arrhythmia [3–5]. According to the literature, the combination of a beta-blocker and non-dihydropyridine CCBs such as verapamil or diltiazem is not recommended, because their additive negative effects on heart rate and atrioventricular (AV) conduction may result in severe bradycardia or heart block [6]. Hence, these antihypertensive agents should be used alone. Furthermore, a common situation in optimal therapy is the lack of universal methods for trace level and quantitative drug analysis in a wide variety of sample matrices. Simultaneous determination of several cardiovascular drug groups is, therefore, highly desirable in the cases of intoxication, controlling the therapy compliance of patients, and pharmacokinetic interactions with other drugs (lipid-soluble beta-blockers with calcium antagonists). For these studies, efficient, selective, and reproducible bioassay methods are, therefore, essential in order to effectively monitor levels and to make proper dose adjustments. Different bioanalytical methods have been reported for antiarrhythmic drug determination in biofluids (including human plasma, whole blood, and urine). These methods include conventional high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) [7,8], tandem

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MS/MS [9–11], fluorimetry (FL) [12,13], and ultraviolet (UV), particularly those in a diode-array configuration detection system [14–17]. Moreover, gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), and capillary electrophoretic (CE) methods [18–20] have also been used. GC–MS analysis for the less or semi-volatile analytes (such as BBs and CCBs) is often accomplished after derivatization to improve their gas chromatographic behavior [21,22]. This step not only complicates the analysis but also increases the time required to analyze these drugs. CE methods suffer from a few problems when compared with HPLC for therapeutic drug monitoring (TDM). The major problem in drug analysis by CE is the sub-optimal detection limits, especially for analysis of drugs that tend to be present in a low concentration [23]. Unlike HPLC, CE is greatly affected by the sample matrix that include salts and proteins which can affect the enhancement of analyte detection in stacking methods [24]. In addition, injection repeatability in CE is generally not as good as that of HPLC. Therefore, improving precision in order to get reproducible data is necessary [25]. However, many of these problems can be solved by using HPLC methods, but most of the published HPLC assays are associated with tedious and time-consuming extraction steps. Hence, for TDM studies, a simple and fast procedure is preferred. Liquid–liquid extraction (LLE) [26], solid-phase extraction (SPE) [27], and protein precipitation (PPT) [28] are the main extraction techniques used to monitor levels of antiarrhythmic drugs in plasma, serum, and urine samples. In addition, salt-assisted liquid–liquid extraction (SALLE) [29], microwave-assisted liquid–liquid extraction (MALLE) [30], solid-phase microextraction (SPME) [31], hollow fiber-liquid phase microextraction (HF-LPME) [32], carrier-mediated liquid phase microextraction (CM-LPME) [33], and exhaustive electromembrane extraction (EME) [34] have also been reported. Recently, some DLLME-based methods combined with spectrofluorimetry [35], HPLC–UV [36,37], CE–MS [38], and FASS–CE–DAD [39] have been reported for the determination of these drugs in human plasma and urine. It should be noted that these methods except the last method were developed for determination of individual drug concentration. In our previous work, low sensitivity limitation associated with CE was improved through the use of hybrid preconcentration methods. LLE is time consuming, labor intensive and requires relatively large quantities of toxic solvents [40]. In most cases, the resulting extract is evaporated to dryness and the residue is reconstituted with a suitable solvent before analysis. SPE is a very popular technique for rapid and selective sample preparation that involves multiple steps such as sorbent conditioning, sample application, washing, and elution. In addition, an extra step, solvent exchange, may be required to preconcentrate the analytes further into smaller volumes [41]. SPE has several advantages when compared with the other sample preparation techniques, such as PPT and LLE. It can be easily automated, gives more efficient separation of interferences from analytes, reduces organic solvent consumption, and is more efficient in analyte recovery. Nevertheless, potential variability of SPE packing, irreversible adsorption of some analytes on SPE cartridges, and more-complex method development are some of the drawbacks of this technique [42]. In order to overcome these problems, microextraction-based techniques can be considered an alternative to the conventional extraction method but all of them have some limitations. In generally, solvent microextraction is defined as an equilibrium-based non-exhaustive sample preparation technique compared with LLE and SPE [43]. In this technique, absolute recoveries are low due to only a portion of the analytes present in the samples that are extracted. In addition, some of these methods require a longer equilibrium time in extraction as compared with DLLME. Thus, DLLME not only reduces equilibration time but also increases the sample throughput within a working day. In this study, the applicability of the DLLME method as an efficient microextraction technique for bioanalysis was assessed. This method was used for analysis of three BBs (metoprolol, propranolol, and carvedilol) and two CCB drugs (diltiazem and verapamil) in human plasma samples. Different factors affecting the DLLME procedure such as type and volume of extraction and dispersive solvents, ionic strength, and sample pH were investigated and optimized. Finally, the developed method was validated according to the Food and Drug Administration (FDA) bioanalytical method validation and then used for the determination of the analytes in real plasma samples. To date, to the best our knowledge, there are no reported studies on the use of DLLME method for simultaneous determination of five antihypertensive agents in plasma samples.

2. Experimental

2.1. Reagents

The reference substances of the studied drugs were kindly supplied by the following Iranian Pharmaceutical Companies: metoprolol by Alborz Darou, propranolol hydrochloride by Rouzdarou, carvedilol by Salehan Chemi, and diltiazem and verapamil by Darou Pakhsh. Molecular structures, log P, p*K*a values, and therapeutic levels of these drugs are reported in Table 1. Acetonitrile (HPLC grade) and methanol, ethanol, acetone, chloroform, dichloromethane, and carbon tetrachloride (analytical grade) were obtained from Scharlau (Barcelona, Spain). Analytical-grade sodium dihydrogen phosphate, sodium chloride, hydrochloric acid (37%, $d=1.18$ g mL^{-1}), and sodium hydroxide were obtained from Merck Company (Darmstadt, Germany). Deionized water was purchased from Shahid Ghazi Pharmaceutical Company (Tabriz, Iran) and was used in this study.

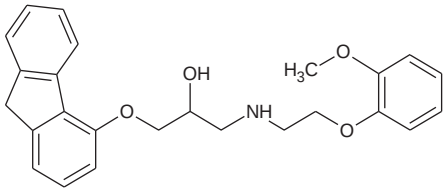
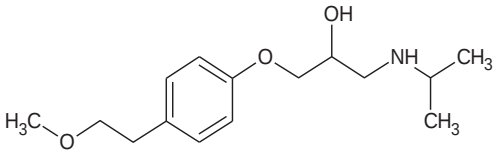
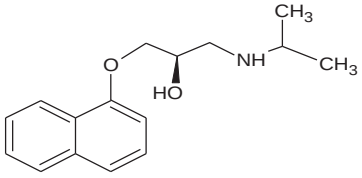
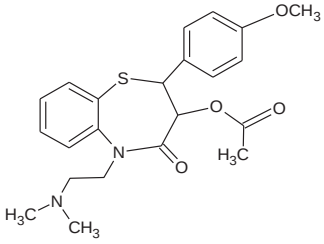
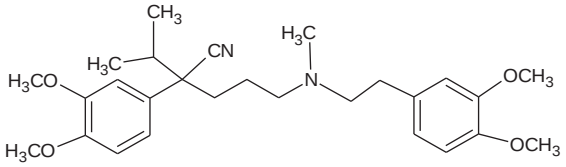
2.2. Preparation of solutions

Individual stock standard solutions (1000 mg L^{-1}) and mixed standard solution (10 mg L^{-1}) of the studied drugs were prepared in methanol. These solutions were stored in a refrigerator at 4 °C for two weeks and used to prepare working solutions in acidified water (pH 3.0). Drug-free (blank) human plasma samples were used for DLLME optimization and method validation of the proposed method, and they were obtained from healthy donors (Iranian Blood Transfusion Research Center, Tabriz, Iran). Human plasma matrix lots were aliquoted into polypropylene microtubes and stored in a freezer at −20 °C. Plasma sample standards that included the therapeutic plasma levels of the studied drugs were prepared daily by dilution of appropriate amounts of the mixed standard solution with the blank plasma. The exact concentrations are found in the text or figures. In addition, real blood samples were collected from cardiac patients who had signed consent forms approved by the ethics committee, Tabriz University of Medical Sciences. These were collected in heparinized tubes and centrifugated (3000 rpm, for 10 min) immediately after collection, and the plasma was separated and stored at −20 °C until assay.

2.3. Chromatographic conditions

HPLC–UV analyses were performed using an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an isocratic pump, a manual sample injector with a 20.0 μL loop (Rheodyne, USA), and a UV variable wavelength detector (VWD). Separations were performed at room temperature on an Agilent ZORBAX Eclipse column (4.6 mm × 100 mm, 3.5 μm particle size) preceded by a guard column (Waters, Milford, PA, USA). ChemStation version B.04.02 (Waldbronn, Germany) was used to

Table 1
Physicochemical properties of the compounds of interest.

Name	Molecular structure	Therapeutic range (μg mL^{-1})	Log *P*	p*Ka*
Carvedilol		0.02–0.16	4.11	7.5
Metoprolol		0.02–0.5	1.79	9.5
Propranolol		0.02–0.3	3.10	9.5
Diltiazem		0.1–0.25	2.7	7.8
Verapamil		0.05–0.25	3.8	8.7

Physicochemical properties calculated using ACD/Labs software version 11.0.

control the HPLC and to process the data. Mobile phase consisted of a mixture of 0.02 M phosphate buffer (adjusted to pH 3.8 with HCl 1 M) and acetonitrile (65:35, v/v). Isocratic elution was performed with a flow rate of 1 mL min^{-1}, and detection wavelength was set at 200 nm.

2.4. DLLME procedure

Drug-free human plasma samples were obtained from healthy volunteers for use as controls and prepared as follows. Overall, 660 μL of blank plasma spiked with the studied drugs (500 ng mL^{-1}) was pretreated by protein precipitation (PPT) clean-up method, and the resultant supernatant was used as dispersant for the subsequent DLLME for further enrichment of the studied drugs. In order to remove high-protein concentrations in plasma samples affecting the HPLC separation, a ratio of 2:1 (precipitant to plasma) was used. For this purpose, 1340 μL of acetonitrile was added to the plasma, vortexed briefly (1 min), and centrifuged at 8000 rpm for 5 min. After that, the colorless supernatant (~1800 μL) resulting from PPT step was mixed with 100 μL dichloromethane and rapidly injected into 5 mL aqueous solution (pH 11.5) containing 1% (w/v) NaCl. After centrifugation at 4000 rpm for 5 min, total sedimented extract (50 μL) was collected and transferred to another microtube after discarding the supernatant. After evaporation of the solvent using an oven at 35 °C, the residue was re-dissolved in 50 μL of acidified water (pH 3) by vortex mixing for 1 min and injected into the HPLC system.

2.5. Parameters and tests for method validation

The consistency, reliability, and accuracy of the analytical results are important in pharmaceutical development for the purpose of preclinical and clinical studies. Therefore, measurements for each analyte in the biological matrix should be validated based on validation guidelines. Method validation studies include all procedures which demonstrate that a method is suitable for its intended use. Therefore, the developed method was validated based on FDA guidelines. In order to do this, the calibration, linearity, lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), intra- and inter-day precisions, accuracy, recovery, selectivity and specificity, stability (room temperature and freeze thaw), and robustness were tested.

3. Results and discussion

3.1. Optimization of DLLME parameters

In order to obtain high enrichment factors (*EF*s) and extraction recoveries (*ER*s), the effect of different parameters such as type

and volume of extracting solvent, type of dispersive solvent, salt addition, pH of sample, and centrifugation time and rate were investigated and optimized using one-variable-at-a-time method. *EF* and *ER* were used to evaluate the performance of DLLME method for the extraction of five antiarrhythmic drugs in plasma. EF is defined as the ratio of final analyte concentration in the sedimented phase (C_{Sed}) after DLLME to the initial concentration of analyte (C_0) in sample solution:

$$EF = \frac{C_{Sed}}{C_0} \quad (1)$$

C_{Sed} is calculated from a calibration graph obtained from a direct injection of the mixed standard solutions of drugs (0.125–50 mg L^{-1}) in acidified water (pH 3). *ER* is the percentage of total analyte amount (n_0), which is extracted to the organic phase (n_{Sed}), and is a function of *EF* and the phase volume ratio (V_{Sed}/V_0).

$$ER = \frac{n_{Sed}}{n_0} \times 100 = \frac{C_{Sed} \times V_{Sed}}{C_0 \times V_0} \times 100 \quad (2)$$

$$ER = \left(\frac{V_{Sed}}{V_0}\right) \times EF \times 100 \quad (3)$$

where V_{Sed} and V_0 are volumes of the sedimented phase and sample solution, respectively.

3.1.1. Selection of extraction solvents

The selection of an appropriate extraction solvent is very important in DLLME in order to obtain an efficient extraction. Extraction solvent is selected on the basis of its density, extraction capability of interested compounds, low solubility in water, and good chromatographic behavior. In addition, its ability for the selective separation of the interest compounds from endogenous interfering components matrix is considered. In this work, three halogenated hydrocarbons, including chloroform, carbon tetrachloride, and dichloromethane, that have these abilities were applied for this purpose. To do this, plasma proteins were precipitated using 1340 μL of acetonitrile and resultant supernatant was used as a disperser solvent in the subsequent DLLME procedure. In order to achieve similar volumes of the sedimented phase (60 μL) after DLLME, colorless supernatant resulting from PPT step was mixed with 100 μL dichloromethane, 80 μL carbon tetrachloride, and 60 μL chloroform. According to the obtained results (Fig. 1), dichloromethane showed higher extraction efficiency for all five studied drugs than those of other extraction solvents. Consequently, dichloromethane was selected as the optimum extraction solvent.

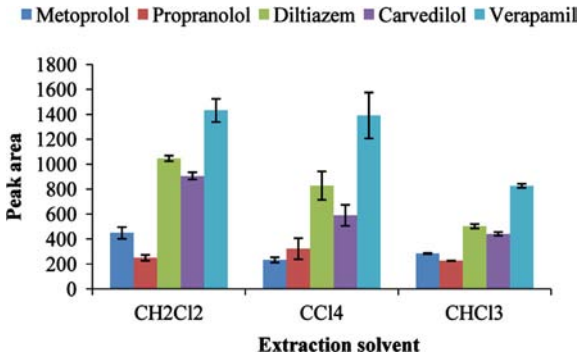


Fig. 1. Optimization of extraction solvent type. Extraction conditions: extraction solvent, CH_2Cl_2 (100 μL), CCl_4 (80 μL), $CHCl_3$ (60 μL); disperser solvent, acetonitrile (1340 μL); concentration of the spiked drugs in plasma, 500 ng mL^{-1}; aqueous sample volume, 5 mL; salt amount, 2.0% (w/v), NaCl; pH, 11.5; rate and time of centrifugation, 4000 rpm for 5 min, respectively. The bars indicate standard deviations ($n=3$).

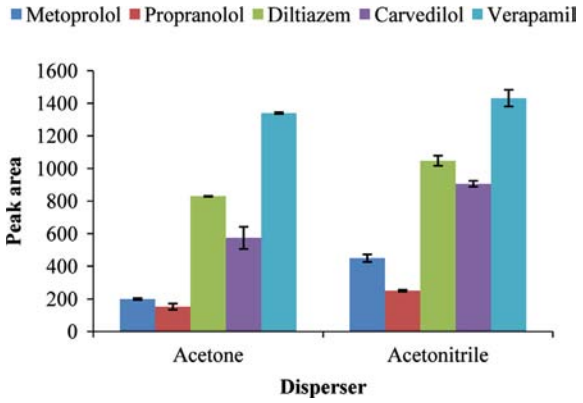


Fig. 2. Optimization of diserser solvent type. Extraction conditions: extraction solvent, CH_2Cl_2 (100 μL); disperser solvent volume, (1340 μL); concentration of the spiked drugs in plasma, 500 ng mL^{-1}; aqueous sample volume, 5 mL; salt amount, 2.0% (w/v), NaCl; pH, 11.5; rate and time of centrifugation, 4000 rpm for 5 min, respectively. The bars indicate standard deviations ($n=3$).

3.1.2. Selection of disperser solvent

Almost all drugs are reversibly bound to a wide variety of plasma proteins. Drug molecules should be dissociated from plasma proteins in total drug analysis. For this purpose, PPT is commonly used to precipitate any protein and solubilize any bound drug. Organic solvents (methanol, ethanol, acetonitrile, or acetone) are the common solutions that are used as protein precipitation agents. Since the supernatant is aqueous, it can be employed as a disperser solvent in DLLME due to appropriate miscibility with both water and the extraction solvent. Spiked plasma samples were pretreated with 1340 μL of each disperser solvent. After PPT process, the supernatant liquid (~1800 μL) was transferred for use as a disperser. With methanol and ethanol, a two-phase system was not formed. However, in the case of acetonitrile and acetone, more stable two-phase systems were observed. Based on the experimental results (Fig. 2), acetonitrile was selected as optimum disperser solvent, because it can recover more drugs from plasma in the PPT step and produce good dispersion (cloudy solution) in the DLLME step. It should be noted that the acetonitrile:plasma volume ratio was not varied, as this ratio was necessary for an efficient protein precipitation.

3.1.3. Effect of extraction solvent volume

The effect of extraction solvent volume on the analytical signals was also investigated. Different volumes of dichloromethane (80, 100, 120, 140, 160, and 180 μL) were added to the resulting supernatant solution (~1800 μL) and subjected to the same DLLME procedure. By increasing the volume of dichloromethane, volume of the sedimented phase increased from 60 to 260 μL. In all cases, a constant volume of the settled phase (i.e. 60 μL) was removed and dried. Then, residue was reconstituted with 50 μL of the acidified water (pH 3.0) before analysis. It should be noted that at low volumes of the extraction solvent (i.e. 80 μL), the two-phase system was not formed and, thus, the method was not applicable. As can be seen in Fig. 3 by increasing extraction solvent volume, peak areas (sensitivity) and, as a result, EFs decrease due to increasing the separated organic phase volume and dilution effect. Thus, 100 μL of dichloromethane was determined to be the optimal extraction solvent volume.

3.1.4. Effect of salt addition

For analytes that are relatively soluble in water, a salting-out agent is used in order to improve the recoveries in liquid–liquid extraction, as it generally makes the analytes less soluble in water due to the salting-out effect. On the other hand, the presence of a

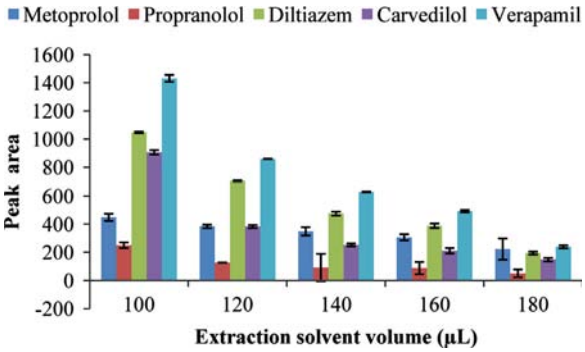


Fig. 3. Optimization of extraction solvent volume. Extraction conditions: extraction solvent, CH_2Cl_2; disperser solvent, acetonitrile (1340 μL); concentration of the spiked drugs in plasma, 500 ng mL^{-1}; aqueous sample volume, 5 mL; salt amount, 2.0% (w/v), NaCl; pH, 11.5; rate and time of centrifugation, 4000 rpm for 5 min, respectively. The bars indicate standard deviations ($n=3$).

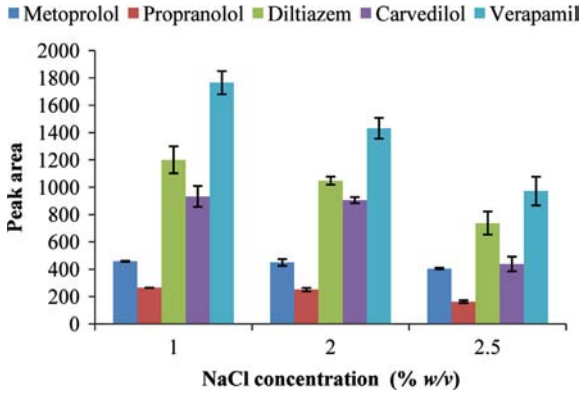


Fig. 4. Optimization of salt concentration. Extraction conditions: extraction solvent, CH_2Cl_2 (100 μL); disperser solvent, acetonitrile (1340 μL); concentration of the spiked drugs in plasma, 500 ng mL^{-1}; aqueous sample volume, 5 mL; pH, 11.5; rate and time of centrifugation, 4000 rpm for 5 min, respectively. The bars indicate standard deviations ($n=3$).

salt in the extraction system promotes the phase separation of extractant after centrifugation. So, in this study, the effect of salt addition on the extraction efficiency was evaluated by adding NaCl into the aqueous solution in the range of 0.0–3.5% (w/v). In the absence of NaCl, after centrifugation, very little turbid sedimented phase was obtained at the bottom of the conical test tube and, consequently, it was not collected. In addition, at high concentrations of NaCl (3 and 3.5% w/v), density of the aqueous phase increased so that extraction solvent floated on the aqueous solution, and, thus, the method was not applicable. In all cases, a constant volume of the settled phase (i.e. 50 μL) was removed and dried. Then, residue was reconstituted with 50 μL of the acidified water (pH 3.0) before injection. By increasing the concentration of NaCl from 1 to 2.5% (w/v), the sedimented phase volume increased due to decreasing solubility of the organic phase in water, which, consequently, resulted in a decrease in concentration of the desired drugs (Fig. 4). On the basis of these results, the concentration of sodium chloride was set at 1.0% (w/v) in the subsequent experiments.

3.1.5. Effect of pH

pH is another important parameter for extraction studies. In order to effectively neutralize the analytes, pH of the solution should be adjusted properly so that additional selectivity can be achieved through adequate control of the pH. All the analytes are basic and have p*K*a values above 7.0 (see Table 1). In practical

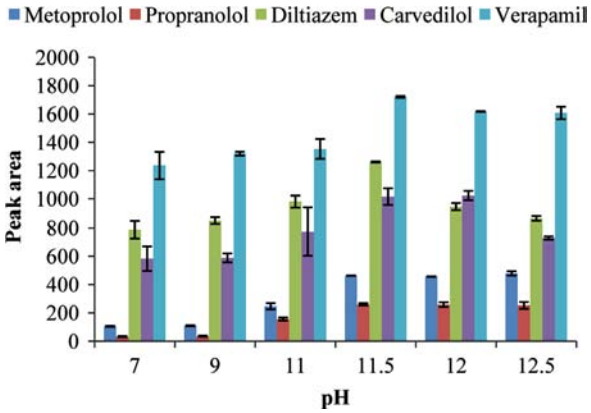


Fig. 5. Optimization of pH. Extraction conditions: extraction solvent, CH_2Cl_2 (100 μL); disperser solvent, acetonitrile (1340 μL); concentration of the spiked drugs in plasma, 500 ng mL^{-1}; aqueous sample volume, 5 mL; salt amount, 1.0% (w/v), NaCl; rate and time of centrifugation, 4000 rpm for 5 min, respectively. The bars indicate standard deviations ($n=3$).

terms, the neutral molecule of bases exists in solutions of pH > p*K*a+2. In order to investigate this, the influence of pH changes on the efficiency of the extraction at 6 pH levels (7–12.5) was studied. As shown in Fig. 5, extraction efficiency for the tested drugs was slightly decreased at pH values lower and higher than 11.5. It can be attributed to protonation of drugs at lower pHs and hydrolysis of drugs under highly alkaline conditions. Hence, pH 11.5 was chosen as the optimum pH for further studies.

3.1.6. Optimization of centrifugation time and rate

In order to finish the DLLME process optimization, the test tube where extraction took place was centrifuged. After centrifugation, the organic droplets containing the target analytes can easily be recovered and subjected to further treatment. To optimize the centrifugation step, both centrifugation time and rate were varied in the ranges of 5–15 min and 2000–6000 rpm, respectively. The obtained results indicated that these parameters were less effective and so, 4000 rpm and 5 min were selected as the optimized centrifuge rate and time, respectively (see Figs. 1S and 2S (of Supplementary information) for details).

3.2. Validation reports

3.2.1. Linearity and calibration curves

For assessment of linearity of the proposed method, the matrix-matched calibration curves were constructed by plotting the peak area versus drug nominal concentrations of the seven plasma standards. Linearity curve's correlation coefficient (r^2), *y*-intercept, slope of the regression line, and their standard errors were computed from the mean of three calibration curves that were prepared in 3 different days. Limits of detection (LOD) and quantification (LOQ) were evaluated on the basis of the signal-to-noise ratio (*S*/*N*) of three and ten, respectively. *S*/*N* was estimated using measurements of the peak height relative to the baseline noise, and height values were consequently converted into concentrations through the height of the analyte peaks at the LLOQ. The LLOQ was reported as the lowest concentration on the calibration curve that could be determined with a precision of RSD $\leq$ 20% and an accuracy of 80–120%. The ULOQ was identical with the highest calibration curve point that could be quantified with acceptable uncertainty. Accordingly, the concentration range between LLOQ and ULOQ was defined as the validated range for the assay. The obtained results are summarized in Table 2.

Table 2
Quantitative results of the proposed method for the selected drugs in plasma samples.

Parameter	Metoprolol	Propranolol	Diltiazem	Carvedilol	Verapamil
Linear range ($\mu g\ mL^{-1}$)	0.02–1	0.02–1	0.02–1	0.02–1	0.02–1
Slope	621	458	1683	1286	2298
Slope standard errors	33.7	13.2	7.6	49.3	57.3
Intercept	14.0	0.8	5.7	17.0	27.8
Intercept standard errors	3.2	4.2	8.3	7.9	10.6
Coefficient of determination (r^2)	0.997	0.999	0.997	0.998	0.998
Number of data points	7	7	7	7	7
LOD[a] ($\mu g\ mL^{-1}$)	0.002	0.005	0.003	0.006	0.003
LOQ[b] ($\mu g\ mL^{-1}$)	0.007	0.018	0.009	0.019	0.010
LLOQ[c] ($\mu g\ mL^{-1}$)	0.02	0.02	0.02	0.02	0.02
EF[d]	4.4	7.9	10.8	7.8	10
ER%[e]	33	60	82	59	76

[a] Limit of detection.
[b] Limit of quantification.
[c] Lower limit of quantification.
[d] Enrichment factor.
[e] Extraction recovery.

Table 3
Precision and accuracy of the method for determination of the studied drugs in plasma samples.

Drug	Nominal concentration ($ng\ mL^{-1}$) ($n=5$)	Intra-assay precision (RSD%) ($n=5$)	Accuracy (RE%)	Inter-assay precision (RSD%) ($n=15$)	Accuracy (RE%)
Metoprolol	50	15	−2.1	12.1	3.7
	500	5.1	0.2	6.6	2.8
	800	5.3	−8.2	8.2	−6.0
Propranolol	50	9.4	−7.4	10.4	3.8
	500	6.9	−2.4	5.8	−0.8
	800	7.6	−12.5	8.3	−6.2
Diltiazem	50	13.4	−2.2	8.2	−0.04
	500	2.6	−7.1	4.9	−5.1
	800	9.8	−13.3	7.3	−7.3
Carvedilol	50	9.7	−14.4	10	−6.8
	500	3.1	−3.7	6.3	−1.4
	800	3.4	−9.5	6.1	−5.6
Verapamil	50	5.4	−12.3	5.5	−9.8
	500	2	−5.5	2.7	−3.1
	800	4.5	−5.5	5.3	−0.3

3.2.2. Precision and accuracy

In this section, precision and accuracy of the method were assessed under the optimized conditions for both intra- and inter-days. These two parameters are expressed as the closeness of the individual measures of an analyte and deviation of mean test results from nominal concentrations, respectively. In order to do this, quality control (QC) samples were prepared at low, medium, and high concentrations of 50, 500, and 800 $ng\ mL^{-1}$ and analyzed on the same day (intra-day assay) and on five different days (inter-day assay). Calculations were based on five replicates (intra-day) and 15 replicates (inter-day) of QC samples. The obtained results were expressed as the relative standard deviation (RSD) and relative error (RE). The obtained results are presented in Table 3. REs were found to be between −14.4 and 3.8%, and RSDs were between 2 and 15%. The results demonstrated that the values were within the acceptable range recommended by FDA, and, hence, the method is sufficiently accurate and precise.

3.2.3. Recovery

An accuracy criterion is usually evaluated based on the spiked recovery approach. This is performed using a minimum of nine determinations over a minimum of three concentrations (low, medium, and high) and reported as relative recoveries (*RR*) and can be calculated as follows:

$$RR = \frac{C_{found} - C_{real}}{C_{added}} \times 100 \quad (4)$$

where C_{found} is the analyte concentration measured from the sample after analyte addition, C_{real} is the native analyte concentration, and C_{added} is the amount analyte that was added to the sample. Table 4 shows the recovery data obtained during method validation. The calculated *RR*s were in the range from 90 to 104% and demonstrate the suitability of the sample preparation method for the analysis of the examined compounds in plasma samples.

3.2.4. Selectivity and specificity

The selectivity and specificity tests should be assessed to show the ability of the bioanalytical method to measure and differentiate the analytes in the presence of other components, either endogenous or exogenous, in sample matrix under the stated conditions of the method. In assays with low intrinsic selectivity (e.g. HPLC with detection other than MS), the selectivity and specificity are derived partly not only from the analysis but also from the initial clean-up process. These are achieved by changing the chromatographic condition (i.e. mobile phase constituents, detector wavelength) and using an optimized sample preparation. In this study, specificity was investigated by analyzing six independent sources of control matrix. Representative chromatograms for blank plasma and the spiked plasma with a concentration of 500 $ng\ mL^{-1}$ of drugs are shown in Fig. 6(A). As can be seen from the chromatograms, no interference was observed in drug-free human plasma samples, which indicates that the optimized sample preparation can eliminate most of the matrix components. In order to check the selectivity of the assay, plasma samples were spiked with the some other drugs (i.e. atorvastatin, atenolol, captopril, enalapril, furosemide, glibenclamide, hydrochlorothiazide, losartan, lovastatin, nifedipine, omeprazole, pantoprazole and sotalol, acetaminophen, caffeine, diazepam, and salicylic acid) at concentrations of 500 $ng\ mL^{-1}$ and analyzed according to the described procedure. Responses of the analytes at the LLOQ concentration were compared with the responses of these spiked samples. No interference from other drugs was observed at the retention time of the studied drugs. These results indicate that the proposed method is specific and selective for the analysis of the desired drugs in plasma samples.

Table 4
Relative recoveries obtained by the proposed method in plasma samples spiked at 50, 500 and 800 ng mL^{-1}.

Drug	Nominal concentration (ng mL^{-1})	Found concentration (ng mL^{-1}) ± SD[a]	Relative recovery (*RR*%) ± SD
Metoprolol	50	52 ± 6	104 ± 13
	500	514 ± 34	103 ± 7
	800	752 ± 62	94 ± 8
Propranolol	50	52 ± 5	104 ± 11
	500	496 ± 29	99 ± 6
	800	750 ± 62	94 ± 8
Diltiazem	50	50 ± 4	100 ± 8
	500	474 ± 23	95 ± 5
	800	742 ± 54	93 ± 7
Carvedilol	50	47 ± 5	93 ± 9
	500	493 ± 31	99 ± 6
	800	755 ± 46	94 ± 6
Verapamil	50	45 ± 2	90 ± 5
	500	484 ± 13	97 ± 3
	800	797 ± 42	100 ± 5

[a] Standard deviation ($n=3$).

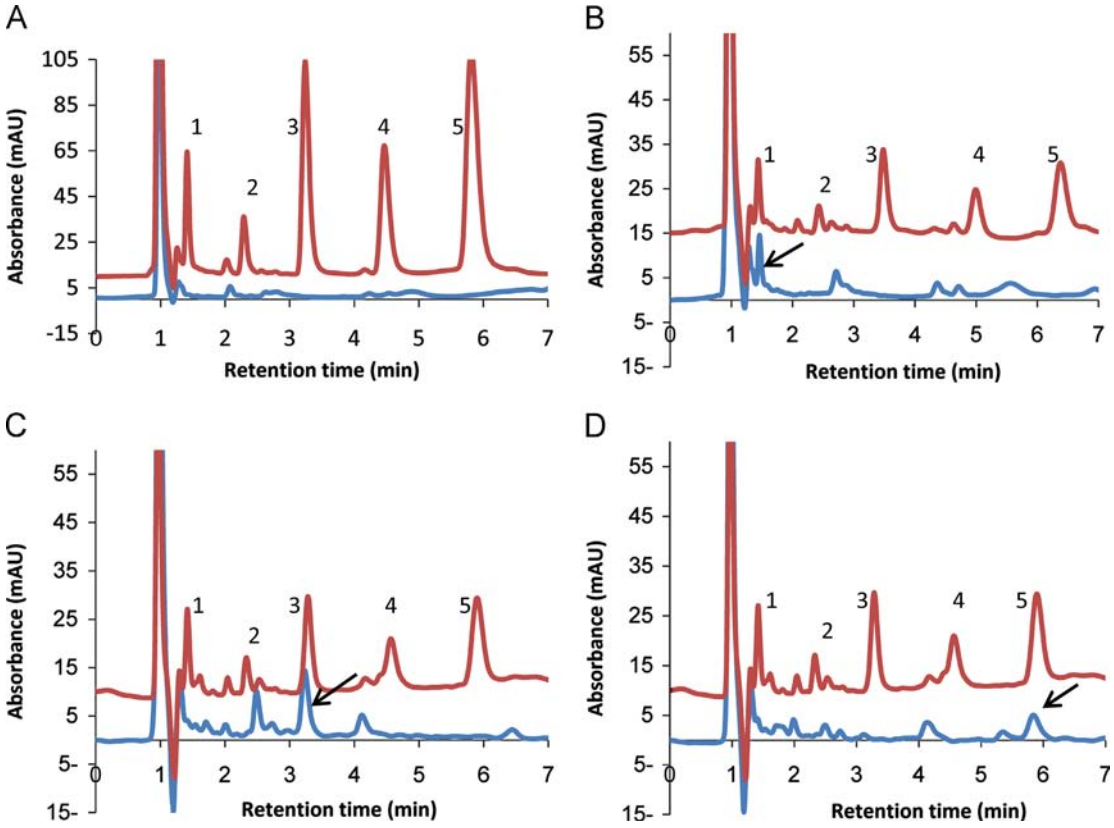


Fig. 6. Typical chromatograms. (A) Chromatograms of blank sample and spiked sample with the selected drugs (each 500 ng mL^{-1}) obtained under optimal DLLME–HPLC/UV (upper is for spiked and lower for blank). (B–D) Chromatograms of real samples and spiked samples with the selected drugs (each 100 ng mL^{-1}) obtained under optimal DLLME–HPLC/UV (upper is for spiked and lower for real samples). Other conditions are the same as those used in Fig. 2S (of supplementary information) for details). Peaks: (1) metoprolol; (2) propranolol; (3) diltiazem; (4) carvedilol; (5) verapamil.

3.2.5. Stability

The drug stability during sample processing and storage is also important in clinical chemistry, in order to interpret drug concentrations accurately. Therefore, stability of the drug(s) in the biological matrix should be investigated under the conditions used for storage and/or processing. In this work, stability assessments were conducted under short-term temperature and freeze and thaw conditions. In order to do these, QC samples were prepared at three different concentration levels (50, 500, and 800 ng mL^{-1}) such as low, medium, and high concentrations, respectively. Three aliquots of each concentration were kept at room temperature for 12 h and analyzed for a room temperature stability study. The freeze–thaw stability of the drugs was also determined after three freeze and thaw (−20 to 25 °C) cycles according to the following conditions. QC samples were frozen at −20 °C, allowed to thaw at room temperature unassisted, and refrozen. The obtained results are shown in Table 1S (Supporting information). REs are between −14.9 and 15%, which confirm the stability of the studied drugs in plasma samples under different storage conditions.

3.2.6. Robustness

Small variations in process parameters can affect the measurement results and should be assessed during method validation.

The reliability of an analysis with regard to deliberate variations in method parameters can be evaluated by robustness testing. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, planned changes to the method conditions. For this purpose, small variations in method parameters are introduced and the quantitative influence of the variables is determined. In this study, the effects of the following changes in separation and extraction conditions were determined: NaCl content in sample solution adjusted by ($\pm 0.2\%$, w/v), sample solution pH adjusted by (approximately ± 0.2 pH units), buffer concentration, and its pH, adjusted by ± 2 mM and ± 0.2 pH units, respectively. Plasma samples were spiked with medium concentration of the analyzed component (500 ng mL^{-1}) and subjected to the DLLME–HPLC/UV procedure under the changed conditions. The relative recoveries ranged from 87 to 106% (see Table 2S (of supplementary information) for details). The obtained results showed that small changes applied in test conditions had no significant effect on the analysis results.

3.3. Comparison of the proposed method with others

Tables 3S and 4S (Supporting information) summarize linear range, LOD, LOQ, extraction/analysis time, matrix type, and sample size of some analytical methods along with the proposed method. As can be seen, several bioanalytical methods were reported for antiarrhythmic drug determination in biological fluids, but most of them were only developed for a number of drugs considered in this study. In most of the cases, sensitive detectors such as MS, tandem MS/MS, electrochemical (EC), electrochemiluminescence (ECL), and FL were used, which are inherently more sensitive than UV. As an overall aspect, higher sensitivity and lower LOD and LOQ values are expected from these methods. These detectors are not actually commercially available and are difficult to fabricate in house, whereas the UV absorption detector is universal (i.e. suitable for many types of analytes) and also has adequate sensitivity for most applications. Moreover, the DAD that was used in some cases provides optimum detection conditions in terms of selectivity and sensitivity in comparison with UV. Since, it provides simultaneous detection of up to 8 compound specific wavelengths for optimum selectivity. Enzymatic hydrolysis, LLE, SPE, and SFE–SPE methods were employed in most cases for clean up or concentration that involve laborious and extensive preparatory procedures before instrumental analysis. Moreover, “quick-and-dirty” sample preparation techniques, such as PPT, or simple “dilute-and-shoot” were used. Although these methods were employed in combination with sensitive instrumental techniques, in most cases, the resulting LODs were comparable to or higher than those of the proposed method. The last two works cited in Table 4S from our group report the analysis of three beta blockers and four antiarrhythmic drugs in urine and plasma samples by capillary zone electrophoresis (CZE)—DAD method coupled with salt-assisted liquid–liquid extraction (SALLE) and DLLME, respectively. These methods require 4 mL of urine and 660 μL of plasma and produce higher and/or comparable LOD values in comparison with the proposed HPLC method. In order to reduce the amounts of toxic organic solvent and inject all the adsorbed analytes into the analytical equipment, some efficient microextraction techniques were recently developed, but some of these methods require longer pretreatment times than our method. Two DLLME-based methods reported in Tables 3S and 4S [37,38] produced higher LOD values for carvedilol in plasma samples and for metoprolol in urine in comparison with the proposed method. It should be noted that in some of the mentioned techniques [36,37], lower LOD values were achieved using both HPLC system and DLLME method. The limited number of quantified analytes, limited linear range [36], and larger required sample size (at least 10 mL of urine) [37] could be considered restriction factors when compared with the proposed method. Hence, the proposed method has several advantages over the other reported techniques, being very simple, rapid, and sensitive enough for antiarrhythmic drug monitoring in human plasma. See also the Figs. 1S and 2S, and Tables 1S–4S [44–52], available at online Supporting information.

Table 5
Determination of the target drugs in patients' plasma samples obtained by the proposed method (results given as mean results, $n=3$).

Nos.	Gender	Age (year)/ weight (kg)	Administered drugs (mg)	Concentration ($\mu g\ L^{-1}$) $\pm$ SD[a]
1	Female	77/68	Metoprolol 50	63.3 ± 4
2	Female	78/86	Metoprolol 100	92.3 ± 4
3	Female	56/59	Metoprolol 100	25.8 ± 2
4	Female	54/75	Metoprolol 50	28.4 ± 2
5	Male	72/70	Metoprolol 25	55.6 ± 3
6	Male	83/61	Diltiazem 120	70.6 ± 2
7	Female	43/100	Verapamil 40	23.6 ± 2

[a] Standard deviation ($n=3$).

3.4. Real sample analysis

The efficiency of the proposed method was further evaluated by determining the concentration of metoprolol, diltiazem, and verapamil in real plasma samples. All patients gave their written consent, and blood samples were collected from patients after oral administration of these drugs individually. The samples were analyzed thrice, and compound identification was performed by comparing the retention times with those of the spiked blank plasma standards. The results of this analysis are shown in Table 5. Representative chromatograms in patients receiving metoprolol, diltiazem, and verapamil are shown in Fig. 6(B–D). Lower chromatograms in Fig. 6(B–D) belong to the patient numbers 1, 6, and 7, respectively. As can be seen, no interference peaks were observed while analyzing these drugs in plasma, which indicate that the method is suitable for clinical studies.

4. Conclusion

In this study, a fast reverse-phase HPLC method with UV detection was developed and validated for the simultaneous determination of five antiarrhythmic drugs (i.e. metoprolol, propranolol, diltiazem, carvedilol, and verapamil) in plasma samples. PPT cleanup and DLLME procedure was used for efficient plasma sample pretreatment. DLLME was shown to be more efficient than widely used extraction and microextraction techniques in bioanalysis. It is simple, rapid, and suitable for high-throughput sample analysis of biological fluids. No significant interferences were observed in plasma samples, indicating that the optimized sample preparation can eliminate most of the matrix components. The high sensitivity of the proposed method enables determination of investigated drugs in spiked human plasma with high specificity/selectivity, accuracy, precision, and reproducibility. Furthermore, the proposed method was successfully applied to the analysis of the selected drugs in real plasma samples at ng mL^{-1} level. Therefore, the presented method can be considered a routine analytical method in drug analysis laboratories for pharmacokinetic, pharmacodynamic, and therapeutic drug monitoring studies.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.12.008.

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