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Capillary electrophoresis with UV detection, on-line stacking and off-line dispersive liquid–liquid microextraction for determination of verapamil enantiomers in plasma

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A rapid, convenient, sensitive and reliable dispersive liquid–liquid microextraction (DLLME) method was coupled with field-amplified sample injection (FASI) in capillary electrophoresis with a diode array detector for the quantification of verapamil enantiomers in human plasma samples. Various parameters affecting the extraction efficiency as well as FASI were optimized. The method performance was studied over the concentration range of 25–350 ng mL^{-1} for each enantiomer in terms of accuracy (recovery = 92–115%), linearity (coefficients of determination (R^2) > 0.99) and repeatability (RSDs% agree within 15%). The method was validated in plasma according to FDA guidelines. This is the first work showing the possibility of the use of DLLME and on-line sample pre-concentration techniques for the analysis of verapamil enantiomers in plasma.

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Introduction

Verapamil or 5[(3,4-dimethoxy phenyl ethyl)methyl-amino]-2-(3,4-dimethoxyphenyl)-2-isopropyl valeronitrile is a member of the calcium channel blocker class and works by relaxing the muscles of the heart and blood vessels. Verapamil is used for the treatment of cardiovascular diseases such as hypertension, angina and arrhythmia.[1] It possesses one asymmetric carbon and therefore exists in two enantiomeric forms (Table 1).

Some receptors and enzymes present stereo-selectivity for different enantiomers of drugs; therefore, the two isomers of verapamil differ in their bioavailability[4] along with their pharmacokinetic[5] and pharmacodynamic effects,[6] with the (*S*)-enantiomer being more active (about 20 times) than the (*R*)-enantiomer.[7] Thus, the development of a rapid, low-cost and efficient analytical enantioseparation method for monitoring verapamil enantiomers has received much attention, and a number of analytical methods have been reported for the determination of its enantiomers in bio-fluids. Table 2 lists these methods along with the employed off-line pre-concentration methods, linear range, LOQ and the reported validation data.

Utilizing CE for the separation of chiral compounds presents some advantages related to sample work-up speed, efficiency and cost. The analysis can be performed by adding desired amounts of chiral selector/selectors to the background electrolyte (BGE). Derivatives of cyclodextrin (CD) are widely used as the selectors due to their abundance, aqueous solubility and reasonable price. Compared to costly and time-consuming chromatographic methods, CE-based enantioseparation methods present benefits such as simplicity and low consumption of sample/reagents. The combination of CE with UV detectors is routinely established in most separation laboratories. One of the most important drawbacks of CE-UV is its short optical path length and small volume of injected sample, which decrease detection sensitivity. Some techniques have emerged to deal with these limitations, such as using extended light path capillaries or providing the more sensitive detectors.

Additionally, in-column pre-concentration techniques have been established as efficient ways to enhance the detection limit of CE, namely, transient isotachophoresis (t-ITP),[17] dynamic pH junction,[18] sweeping,[19] large volume sample stacking (LVSS)[20] and field-amplified sample injection (FASI).[21] FASI relies on the mismatch in ionic strength between the sample matrix and BGE, resulting in a difference in conductivity that concentrates the analyte in a sharp zone. This approach can decrease detection limit for drug monitoring in biological samples without any special modification of the instrument. In order to provide the necessary conductivity difference between the sample and BGE, the sample is usually prepared in a low-conductivity matrix.

The determination of analytes in biological samples must employ a selective, sensitive, precise and accurate preparation method. Due to the high protein content of plasma and the

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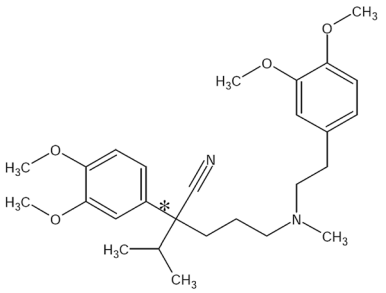
Table 1 Molecular structure and physicochemical parameters of verapamil[a]



Therapeutic range (ng mL^{-1})	50–250 (ref. 2)
log *P*	3.8 (ref. 3)
pK_a	8.9 (ref. 3)

[a] *indicates the chiral center.

subsequent clogging risk in the capillary column, direct injection is not recommended in CE. Additionally, as described above, the preparation of the sample in a low-conductivity matrix enhances the sensitivity of FASI.

Traditional sample preparation methods such as liquid–liquid extraction (LLE) usually suffer from disadvantages such as time-consuming setup, consumption of large quantities of toxic solvents and solvent evaporation (in automation with some analytical instruments).[22] Solid phase extraction involves several steps such as conditioning, sorbent washing and desorption of analytes from the cartridges, which are recommended for single use only.[23]

Current studies are focused on miniaturization (minimizing time and solvent consumption) as well as simplification of the sample preparation step. Dispersive liquid–liquid microextraction (DLLME), which is based on a ternary phase solvent system, is one of the latest modes of liquid-phase extraction. The contact surface between the sample and extraction solvent in DLLME is enlarged by the rapid injection of a mixture of extraction and dispersive solvents into the sample solution.[24] DLLME has some merits including fast operation, no need for large amounts of hazardous solvents, low cost and easy coupling to most analytical instruments.

The purpose of the present work is to develop and validate an easy, inexpensive and efficient CE method for the determination of verapamil enantiomers in plasma samples. The present paper reports the optimization of the experimental conditions that affect the DLLME procedure. Additionally, the applicability of FASI is evaluated. Finally, the DLLME-FASI-CE method is validated for the analysis of verapamil enantiomers in plasma according to FDA guidelines.

Experimental

Chemicals & solutions

Racemic verapamil powder was purchased from Sobhan Darou Company (Rasht, Iran). Acetonitrile (ACN), methanol, acetone, tetrahydrofuran (THF) and chloroform ($CHCl_3$) were obtained from Scharlau (Barcelona, Spain). Sodium hydroxide, orthophosphoric acid, carbon tetrachloride (CCl_4), dichloromethane (CH_2Cl_2) and triethanolamine (TEA) were purchased from Merck (Darmstadt, Germany). Zinc sulfate was purchased from Ajax Chemicals (Auburn, NSW, Australia). Carboxymethyl-β-cyclodextrin (CM-β-CD) was purchased from Fluka Chemicals (Buchs, Switzerland). All reagents and solvents were of analytical grade. Deionized (DI) water (Shahid Ghazi pharmaceutical Company, Tabriz, Iran) was used for sample and BGE preparations. Verapamil stock solution was prepared by dissolving the appropriate amount of racemic drug in methanol to obtain a 1000 μg mL^{-1} solution, and the desired concentrations of drug

Table 2 Some previous studies for the determination of verapamil enantiomers in biological fluids[a]

Method	Sample type/size	Sample preparation	Linear range single enantiomer (ng mL^{-1})	LOQ single enantiomer (ng mL^{-1})	Validation	Ref.
HPLC-fluorescence	Plasma/0.5 mL	LLE	10–85	—	Inter-day and intra-day studies	8
CE-UV using TM-β-CD as a selector	Plasma/1 mL	LLE	2.5–250	2.5	Selectivity, linearity, precision and accuracy	9
HPLC-fluorescence	Plasma/1 mL	LLE	3–200	3	Linearity, accuracy, precision and specificity	10
HPLC-fluorescence	Plasma/1 mL	LLE	2.5–100	—	Inter-day and intra-day studies	11
LC-ESI-MS-MS	Plasma/0.5 mL	LLE	0.1–2.6, 0.1–10.2, 10.2–213	0.1	—	12
HPLC fluorescence	Urine/0.5 mL	SPE	2.5–300	—	Intra-day and inter-day reproducibility and recovery	13
LC-ESI-MS-MS	Plasma/50 μL	LLE	1–250	—	—	14
LC-MS/MS	Rat plasma/100 μL	LLE	1–100	0.5	Precision, accuracy, recovery and stability	15
CE-UV using TM-β-CD as a selector	Plasma/0.5 mL	LLE	250–10 000	200	Precision, accuracy of intra- and inter-day analysis, selectivity and recovery	16

[a] LLE: liquid–liquid extraction, TM-β-CD: trimethyl-β-cyclodextrin.

were prepared by diluting the stock solution with methanol. Buffer composed of 100 mM phosphoric acid adjusted to pH 2.5 with TEA was prepared fresh daily. The BGE used in the study was prepared by dissolving 12 mg CM-β-CD as a selector in 1.5 mL of buffer containing 30% methanol. Drug-free QC plasma samples were provided by Iranian Blood Tranfration Research Center (Tabriz, Iran) and frozen in polypropylene microtubes at −20 °C. Verapamil-spiked plasma samples were also freshly prepared.

Instruments

All experiments were performed using an Agilent 7100 CE (Waldbronn, Germany) system coupled with an online DAD. Instrumental control and data analysis were performed using Agilent Chemstation software (Waldbronn, Germany). The separations were carried out in an uncoated fused-silica capillary (50 μm i.d. and 50 cm total length; 41.5 cm effective length) purchased from Agilent Technology (Waldbronn, Germany). A vortex from Labtron Company (Tehran, Iran) was used in sample preparation. A Sigma centrifuge (Osterode, Germany) was used in the protein precipitation step, and a Hettich centrifuge (Tuttlingen, Germany) was used for sedimentation of the extraction solvent during sample preparation. The pH was adjusted using a Meterohm® pH meter (Herisau, Switzerland). An Alex machine (Istanbul, Turkey) was used to study ultrasonic performance.

Electrophoretic procedure

A new capillary was washed sequentially with 1.0 M NaOH (30 min), DI water (30 min) and BGE (30 min). Between separation runs, the capillary was treated with NaOH 0.1 M (2 min), DI water (2 min) and BGE (5 min). In order to determine the FASI performance, samples were prepared in a 50% water–ACN mixture. The BGE composition was 100 mM of phosphoric acid–TEA buffer at pH 2.5 containing 0.8% (w/v) CM-β-CD and 30% methanol (v/v). All samples and buffers were stored at 4 °C and filtered through a PTFE filter with a pore size of 0.20 μm (Chromafil, Germany). The samples were introduced into the capillary *via* electrokinetic injection at 15 kV for 30 s. A short plug of DI water (50 mbar for 1 s) was loaded before sample injection. The capillary was thermostated at 15 °C. The applied voltage was 25 kV, and on-line UV detection was carried out at 200 nm, the wavelength of maximum sensitivity.

Sample preparation and dispersive liquid–liquid microextraction

Drug-free plasma (400 μL) was left at room temperature for 20 min to thaw and spiked with 0.5 μg mL^{-1} of the racemic verapamil. Acetone (800 μL) was added to the microtube containing spiked plasma and vortexed for 40 s followed by centrifugation at 12 470 × *g* for 5 min to precipitate the proteins.

The yellowish supernatant (1 mL) was placed in a 10 mL glass conical-bottom tube and then diluted with 8 mL of aqueous solution (pH 11.0 adjusted by 1.0 M NaOH) to ensure that the analyte was in its neutral form. The DLLME procedure was performed by quickly injecting 120 μL chloroform (extraction solvent) and 500 μL acetone (dispersive solvent) into the aqueous sample using a 2 mL syringe. A high-turbulence solution was formed immediately as a result of dispersing chloroform within the solution. The targeted analyte was extracted into tiny droplets and collected by centrifugation at 2307 × *g* for 5 min. After discarding the upper aqueous solution, the organic phase was withdrawn with a pipettor and transferred to a microtube for evaporation under N_2 stream. The residue was reconstituted in 100 μL ACN–water (50/50 (v/v)) and vortexed (1 min) for subsequent analysis with CE-UV.

Results and discussion

Optimization of CE-UV system

In order to obtain the chiral resolution in CE, the first step is optimizing the chiral selector concentration. Several concentrations of CM-β-CD ranging from 0.2–1% (w/v) were examined in phosphoric acid–TEA buffer (100 mM, pH 2.5). At low concentrations of selector, the enantiomers were not resolved. The best resolution was achieved at 0.8% CM-β-CD; at higher concentrations of selector, the resolution decreased (Fig. 1).

Enantioseparation shows significant dependency on pH, particularly because of the effect of pH on electro-osmotic flow (EOF). Low pH gives slow EOF, and the analyte migrates through its electrophoretic mobility. Verapamil is a basic drug; therefore, it is fully protonated in acidic media. The separation pH was examined in the range of 2.5–3.5 by employing phosphoric acid–TEA buffer. The ideal separation occurred at pH 2.5. At this pH, CM-β-CD exists in its neutral form (pK_a 4.36), while verapamil is mostly charged.

Buffer concentration (50, 75, 100 and 120 mM) was tested, and the value of 100 mM was selected. The observed current was ≈13 μA. When the concentration was higher than 100 mM, peak shape distortion occurred.

The effect of temperature on the separation could be linked to a number of parameters including the viscosity of BGE and the extent of interaction between analyte and selector. The effect of temperature was investigated in the range of 15–20 °C. Decreasing the temperature to 15 °C increased the migration time and improved the resolution. Thus, the instrument was set at 15 °C.

To evaluate the influence of organic solvents on the resolution, different amounts of methanol (5–35% (v/v)) were added to BGE. Increasing the methanol percentage to 30% increased the

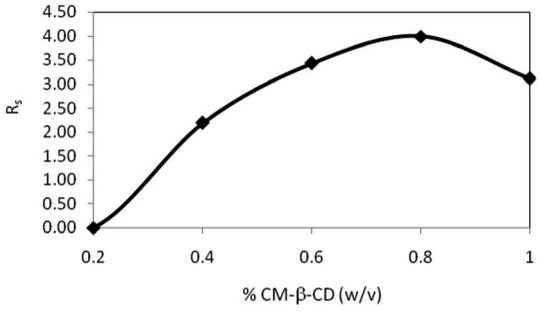


Fig. 1 The effect of chiral selector concentration on the resolution of verapamil enantiomers.

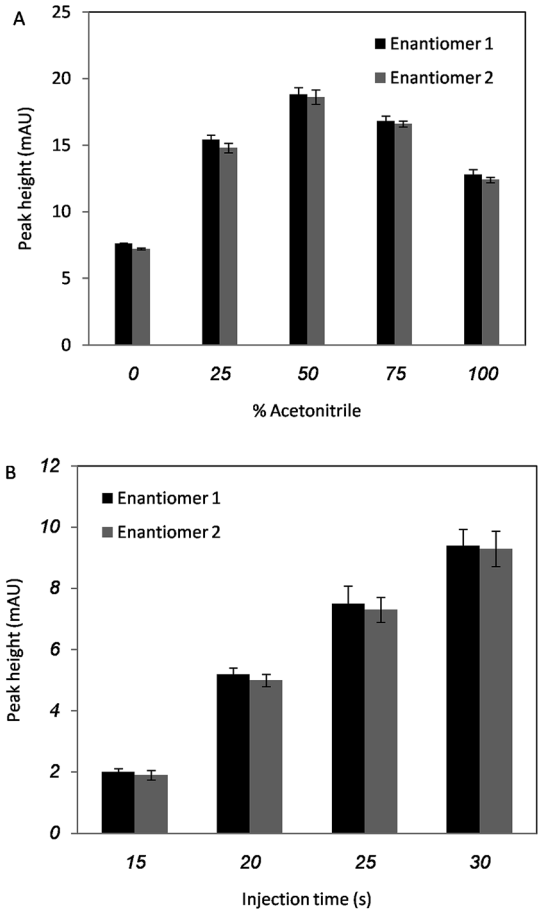


Fig. 2 Optimization of the FASI injection parameters. Separation conditions: uncoated fused-silica capillary, 50 cm (effective length 41.5 cm) × 50 μm i.d.; BGE, 100 mM phosphoric acid–TEA (pH 2.5) containing 30% methanol and 0.8% (w/v) CM-β-CD; detection, UV at 200 nm; temperature, 15 °C; applied voltage, 25 kV. Optimization of (A) sample matrix composition (sample solution: 1 μg mL^{-1} of racemic drug injected at 15 kV for 30 s) and (B) sample injection time (sample solution: 0.5 μg mL^{-1} of racemic drug injected at 15 kV after preliminary pressure injection of water (50 mbar for 1 s)). The error bars indicate the SD ($n = 3$). BGE: background electrolyte; TEA: triethanolamine; CM-β-CD: carboxy methyl beta cyclodextrin; FASI: field-amplified sample injection.

resolution. Further increasing the percentage of methanol to 35% decreased the resolution. Therefore, 30% (v/v) methanol was selected for further experiments.

On-line pre-concentration: FASI-CE

In order to obtain acceptable levels of sensitivity, FASI was used for on-line sample preconcentration. FASI is performed by preparing the sample in a less conducting media than BGE. When the voltage is applied, the sample zone migrates faster until it reaches the zone with high conductivity (lower electric field strength), and resulted in more concentrated analytes. Several parameters must be optimized for the proper execution of FASI, including the composition of the sample matrix, injection time, voltage and the presence/absence of a high-resistivity plug before the introduction of the sample.

Table 3 Details of protein precipitation optimization on a 0.5 μg mL^{-1} spiked verapamil sample

Precipitant	Precipitant : plasma ratio (v/v)	Peak height for enantiomer 1
ACN	0.5 : 1	5.6
	1 : 1	6.1
	2 : 1	9.4
	3 : 1	4.2
Acetone	0.5 : 1	6.9
	1 : 1	7.3
	2 : 1	10.5
	3 : 1	4
ACN + zinc sulfate (1 M)	0.5 : 1	4.3
	1 : 1	5
	2 : 1	3.2
	3 : 1	2.3
Acetone + zinc sulfate (1 M)	0.5 : 1	3.6
	1 : 1	3.1
	2 : 1	2.2
	3 : 1	2.9

The mobility of the analyte is affected by the dielectric constant and viscosity of the sample solvent.[25] To investigate the effect of sample matrix on FASI, standard racemic verapamil solutions (1 μg mL^{-1}) were prepared in various high-resistivity media: diluted BGE (ten-fold), DI water, ACN and its corresponding binary 25/75, 50/50 and 75/25 (v/v) mixtures with DI water. The analytes were not detected when they were dissolved in diluted separation BGE. As can be seen from Fig. 2A, the signal for the analytes significantly increased in 50% ACN.

The FASI performance could be further improved by loading a plug of water or another high-resistivity solvent prior to sample injection.[26] A preinjection water plug was loaded at 50 mbar for 0 to 3 s. Injection times exceeding 1 s disrupted the resolution. Thus, injecting a water plug at 50 mbar for 1 s helps to slightly increase the signal intensity.

Injection time (15–30 s) and injection voltage (10–20 kV) were checked. Based on the values of signal as well as resolution, a voltage of 15 kV was selected. As shown in Fig. 2B, the best signal amplification was observed for electrokinetic injection at 15 kV for 30 s. Injection times longer than 30 s resulted in unresolved peaks.

In summary, the optimum conditions for separation and stacking were as follows: 100 mM phosphoric acid–TEA (pH 2.5) containing 30% methanol and 0.8% CM-β-CD (w/v). Injection was performed for 30 s at 15 kV after the injection of a water plug at 50 mbar for 1 s while temperature was kept at 15 °C. The applied voltage was 25 kV. Samples were prepared in a mixture of 100 μL ACN–water (1 : 1 (v/v)).

Investigation of the protein precipitation step

Protein precipitation processing is necessary for fast clean-up as well as protein-drug binding cleavage in plasma.[27] Plasma samples (400 μL) were spiked by 0.5 μg mL^{-1} of racemic verapamil in a 2 mL polypropylene microtube and left for 20 min at room temperature. The simplification of the complex

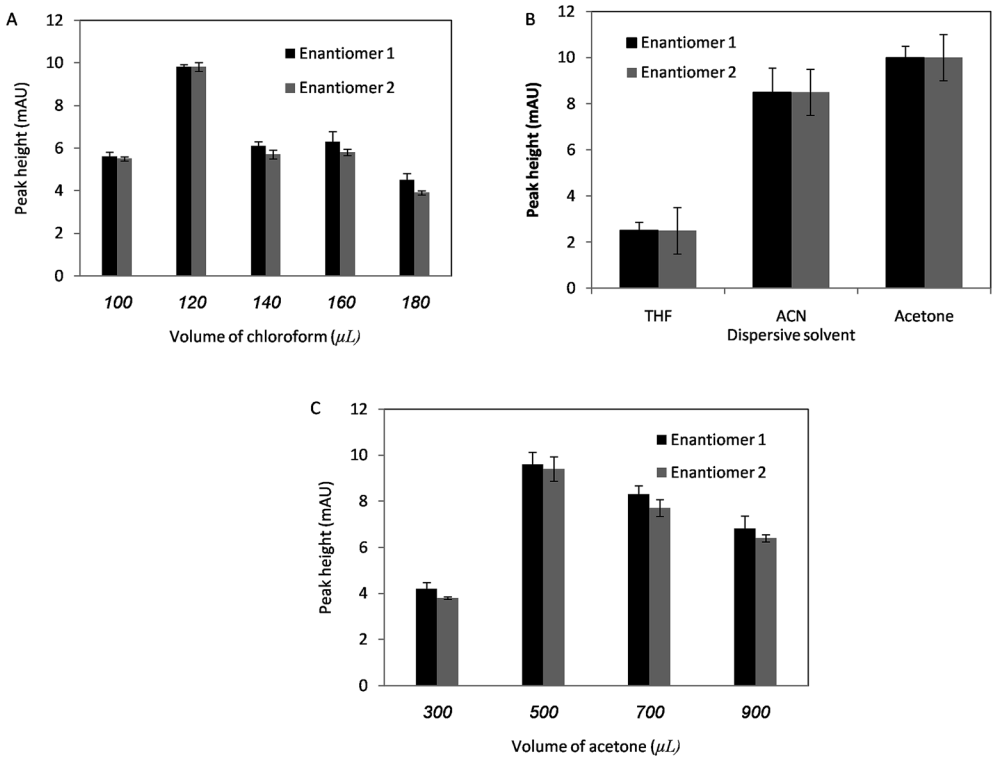


Fig. 3 Optimization of extraction procedure: (A) extraction solvent volume; (B) dispersive solvent type; and (C) dispersive solvent volume. Extraction conditions: concentration of the spiked racemic verapamil in plasma, 500 ng mL^{-1}; aqueous sample volume, 9 mL; rate and time of centrifugation, 2307 × *g* for 5 min. The pH was adjusted to 11 by NaOH (1.0 M). The error bars indicate the SD ($n = 3$).

plasma matrix with organic solvents was tested in the presence of various solvents including acetone, ACN, acetone + zinc sulfate (1 M) and ACN + zinc sulfate (1 M) in a ratio of 90 : 10 (v/v). Each precipitant was added to spiked plasma in the volume ratios of 0.5 : 1, 1 : 1, 2 : 1 and 3 : 1 (precipitant–plasma ratio (v/v)). The solutions were vortexed for 40 s and then centrifuged for 5 min at 12 470 × *g*. The solutions were then subjected to the described DLLME procedure and then analyzed using CE. The corresponding data for the investigation of the appropriate precipitant are shown in Table 3.

Protein precipitation was performed by the addition of 800 μL of acetone to 400 μL of plasma as this resulted in the highest signal obtained by the detector.

Optimization of extraction solvent: type and volume

To achieve an efficient extraction, the extraction solvent should possess some specific characteristics. Its density should be higher than water in order to collect the sedimented phase at the bottom of the conical test tube. It should solubilize the target analyte while leaving the matrix intact. Due to the incompatibility of most solvents with capillary columns (such as halogenated solvents) and considering the extract concentration, the ease of evaporating the solvent should be taken into account. Chlorinated solvents such as $CHCl_3$, CCl_4 and CH_2Cl_2 are good choices. By applying 100 μL of each solvent in the DLLME procedure, the effect of extraction solvent was evaluated. No sediment phase was observed when 100 μL of CH_2Cl_2 was applied as an extraction solvent; this was probably attributed to its higher solubility in aqueous solution. The corresponding signals obtained using CCl_4 are 7.5 ± 0.1 and 7.4 ± 0.1 for the first and second enantiomers, respectively. When $CHCl_3$ was used, the obtained signal was 7.9 ± 0.1 for both verapamil enantiomers. Consequently, the best signal was achieved by $CHCl_3$, which was employed as the extraction solvent in subsequent studies.

Different volumes of chloroform (100, 120, 140, 160 and 180 μL) were added to 500 μL of acetone followed by DLLME to determine the effect of chloroform volume. As shown in Fig. 3A, the signal intensity of the verapamil enantiomers increased with increasing chloroform volume up to 120 μL. Hence, 120 μL was selected for further analyses.

Optimization of the method of emulsion formation

The generation of tiny droplets to increase the contact area between the extraction solvent and aqueous phase is the key step in DLLME. A cloudy solution can be formed using a dispersive-solvent-free method (*e.g.*, ultrasound-,[28] vortex-[29] or air-assisted[30]). The purpose of these methods is to promote the turbidity, which increases the contact surface area and results in the mass transfer of the target analyte from the sample solution to the organic phase. In practice, the formation of a cloudy state is followed by several methods such as sonication-, vortex- and air-assisted microextractions instead of using dispersive solvent. Chloroform (120 μL) as an extraction solvent

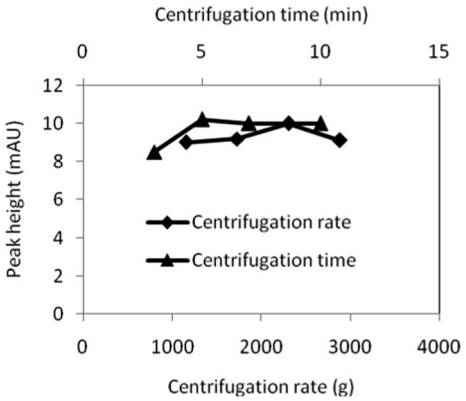


Fig. 4 Optimization of centrifugation rate and time. Other conditions are the same as in Fig. 3.

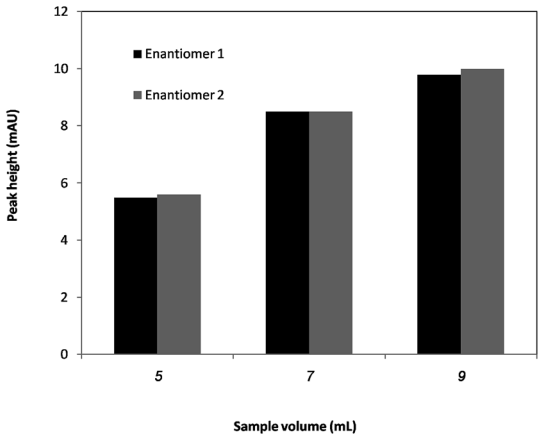


Fig. 5 Optimization of sample volume. Other conditions are the same as in Fig. 3.

was introduced in the conical tube containing sample solution prepared as described in the sample preparation section. The tube was immersed in an ultrasonic water bath, and extraction was performed for 10 min at 25 °C. In another setup, to investigate the effect of shaking, the sample mixture was vigorously shaken using a vortex for 2 min.

Finally, to evaluate the aid of air in the extraction procedure, the sample mixture was rapidly sucked into a 5 mL syringe and then injected into the tube (five times) *via* syringe needle.

After centrifuging the obtained cloudy solutions, the white floccus phase settled on the interface of the two phases, making it difficult to collect the organic phase.

Compared to ultrasonic liquid–liquid microextraction setup, the DLLME approach requires simple laboratory equipment and can be performed in a couple of minutes. Most reported dispersive-solvent methods, such as those tested in the present work, employ aqueous solutions, whereas samples of biological origin exhibit completely different behaviors. Utilizing other dispersion methods instead of dispersive solvents in biological samples has been seldom reported. In complex matrices, recovering the organic phase after dispersion into the sample with the aid of any dispersion agent is challenging. Therefore, the merit of each alternative technique to the use of dispersive solvents for increasing the contact between analyte and extraction solvent should be evaluated based on the degree of organic phase recovery.

In the present study, in addition to increasing the extraction time, the alternative methods resulted in lower efficiencies. Thus, the use of dispersive solvent was adopted for further experiments in this work.

Optimization of dispersive solvent: type and volume

The dispersive solvent should be miscible in both extraction solvent as well as in the aqueous phase to produce fine droplets of extraction solvent.

The sample solution consisting of diluted plasma + acetone (added as a protein precipitant) was treated with a mixture of 120 μL chloroform and different dispersive solvents (methanol, acetone, ACN and THF) and analyzed as described previously in the DLLME performance section. A two-phase system was formed well for all solvents except methanol. Acetone formed a good dispersion solution and gave a high signal (Fig. 3B); therefore, acetone was selected as the dispersive solvent in further analyses. The volume of dispersive solvent is an important parameter that exhibits a crucial effect on extraction efficiency. Different volumes of acetone (300, 500, 700 and 900 μL) were investigated. As can be observed in Fig. 3C, the analyte signal increased with increasing acetone volume up to 500 μL and then decreased with further increases. Thus, 0.5 mL of acetone was chosen as the appropriate volume of dispersive solvent.

Optimization of centrifugation time and rate

DLLME finishes with centrifugation in order to recover the organic phase droplets containing target analyte at the bottom of the tube. To monitor this step, the centrifugation rate and time were studied in the ranges of 1153–2883 × *g* and 3–10 min, respectively, revealing no significant effect on peak height (Fig. 4); so, 2307 × *g* for 5 min were chosen as optimum values.

Sample dilution effect

Due to the complexity of plasma media, matrix effects are suspected. Therefore, the effect of sample volume on extraction

Table 4 Quantitative results of DLLME-FASI-CE-UV for verapamil enantiomers in plasma samples

Analyte	Calibration curve	R^2	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	LLOQ (ng mL^{-1})
Enantiomer 1	$Y = 42.57x - 0.13$	0.996	25–350	15	25
Enantiomer 2	$Y = 41.31x - 0.02$	0.997	25–350	15	25

Table 5 Assay precision and accuracy of QC samples spiked with verapamil

Analyte	Nominal concentration (ng mL^{-1})	Intra-day RSD%; $n = 5^a$	Inter-day RSD%; $n = 5^b$	Accuracy^c (RE%)
Enantiomer 1	25	10	14	15
	150	3	12	−7
	250	4	15	−1
Enantiomer 2	25	11	12	8
	150	3	13	−4
	250	4	15	−1

^{a} Number of replicates. ^{b} Number of days. ^{c} RE% = 100 × ((found value − nominal value)/nominal value).

efficiency was investigated in the volumes range of 5 to 9 mL. Plasma (1 mL) after spiking and protein precipitation was used in all studied volumes. The analytical response increased upon 1 : 9 (sample–aqueous phase (v/v)) dilution. The results of the optimization are shown in Fig. 5.

Method validation

The linearity of detector response, recovery, accuracy, precision, LOD, sensitivity, selectivity, stability (room temperature and freeze–thaw) and method robustness were evaluated according to the FDA guidance for bioanalytical method validation.[31]

Linearity and calibration curves

Table 4 summarizes the linearity and sensitivity of the proposed method for the target drug. Calibration plots were constructed using spiked plasma samples treated with racemic verapamil following the optimized DLLME procedure in the specific concentration range of 50–700 ng mL^{-1}. The linear range of the calibration curve is wide enough to cover the therapeutic range. A signal-to-noise ratio of 3 : 1 was used to determine LODs. The upper limit of quantification (ULOQ) and LLOQ are the highest and lowest calibration curve points with acceptable uncertainty, respectively. LLOQs cover the expected value for therapeutic drug monitoring purposes.

Precision, accuracy and recovery

Precision was used to compare the uncertainty between different measurements and expressed as %RSD. The RSD% of each level was required to be within the range of 15% for all concentration levels with the exception of the lowest calibration level, which was required to be within 20%.

Accuracy represents the closeness of a measured value to the actual (nominal) value. It is defined as:

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

Accuracy and precision were determined by analyzing three different levels of QC samples, $n = 5$ for each QC level, daily (inter-day) and over five days (intra-day). The accuracy of all QC samples was required to be within the range of ±20% for the LLOQ and ±15% for all other QC levels.[31] Both inter- and intra-day precisions and accuracies were determined. The obtained results are listed in Table 5. The results demonstrated that the values were within the acceptable range, and that the method was accurate and precise.

Recovery calculations were also carried out to demonstrate accuracy. To evaluate the recoveries, three different levels of spiked racemic plasma samples were subjected to DLLME-FASI-CE-UV analysis. The relative recoveries (RR%) obtained using the optimized sample preparation method were calculated using the following equation:

$$\mathrm{RR}\ (\%) = \left(\frac{100 \times \text{Measured value}}{\text{Nominal value}}\right)$$

The calculated recoveries were in the range of 92–115%; this demonstrates the suitability of the sample preparation method for the analysis of verapamil enantiomers in plasma samples. Details of the recovery calculations are presented in Table 6.

Table 6 Recoveries for the extraction and analysis of verapamil in spiked plasma samples using DLLME-FASI-CE-UV

Analyte	Nominal concentration (ng mL^{-1}; $n = 5$)	Mean found concentration (ng mL^{-1})	Mean recovery (%)
Enantiomer 1	25	28	115
	150	139	92
	250	249	99
Enantiomer 2	25	27	108
	150	143	95
	250	249	99

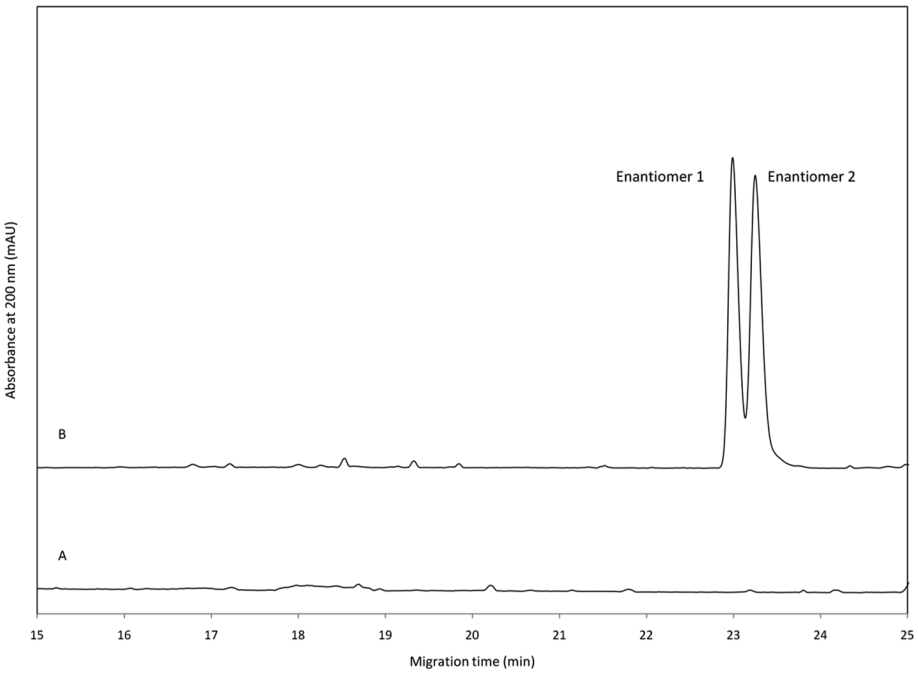


Fig. 6 Typical electropherograms of (A) blank plasma and (B) spiked plasma with racemic verapamil (0.7 μg mL^{-1}).

Specificity and selectivity

The specificity of the method was determined by introducing the blank sample, which was prepared in the manner described previously. The method is specific as no interfering peaks are present in drug-free plasma. Fig. 6 displays the electropherograms of drug-free plasma and spiked plasma under optimal DLLME-FASI-CE-UV. To determine the selectivity of the assay, plasma samples spiked with some other drugs (*i.e.*, losartan, sotalol, diltiazim, salicylic acid, carvedilol, atenolol, diazepam, amiodarone, amiloride, nifedipine, acetaminophen, furosmaide, hydrochlorothiazide and propranolol) were extracted using DLLME, injected into CE using the same conditions at concentrations of 0.5 μg mL^{-1} for each analyte, and analyzed according to the proposed procedure. At the migration time of verapamil enantiomers, no overlap peak was found. The spiked

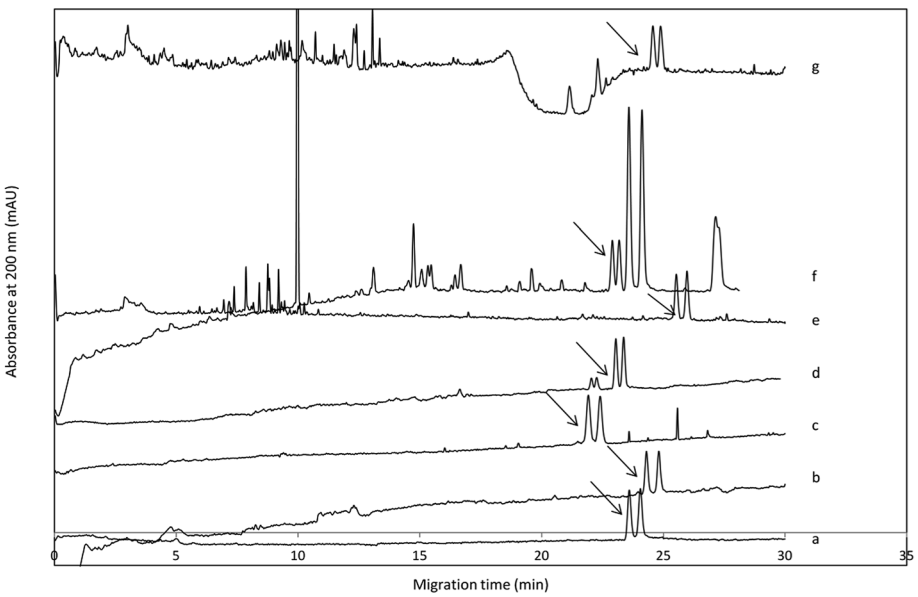


Fig. 7 CE electropherograms in the presence of spiked racemic verapamil (0.05 μg mL^{-1}) with different drugs subjected to the DLLME-FASI-CE procedure: (a) atenolol, (b) sotalol, (c) diazepam, (d) carvedilol, (e) salicylic acid, (f) propranolol and (g) amiodarone. Arrows indicate the verapamil enantiomers.

Table 7 Evaluation of method stability for the extraction and determination of verapamil enantiomers in QC human plasma

		Freeze–thaw stability		Room temperature stability	
Analyte	Nominal concentration (ng mL^{-1}; $n = 3$)	Mean found concentration (ng mL^{-1})	Accuracy (%RE)	Mean found concentration (ng mL^{-1})	Accuracy (%RE)
Enantiomer 1	25	29 ± 1	19	26 ± 1	5
	150	139 ± 12	−7	160 ± 15	6
	250	284 ± 11	13	284 ± 11	13
Enantiomer 2	25	27 ± 4	10	24 ± 3	−1
	150	123 ± 13	−7	143 ± 14	−4
	250	171 ± 15	8	266 ± 12	6

Table 8 Results of the evaluation of method robustness for six different levels[a]

Analyte	Level	Nominal concentration (ng mL^{-1}; $n = 3$)	Mean found concentration (ng mL^{-1})	Accuracy (RE%)	Recovery (%)
Enantiomer 1	1	150	141	−5	94
	2	150	160	7	106
	3	150	139	−7	92
	4	150	139	−7	92
	5	150	162	8	108
	6	150	139	−7	92
Enantiomer 2	1	150	133	−10	89
	2	150	148	−1	98
	3	150	126	−15	84
	4	150	128	−14	85
	5	150	148	−1	98
	6	150	155	3	103

[a] 1: Buffer pH = 2.4, buffer concentration: 98 mM, pH of aqueous solution: 10.5. 2: Buffer pH = 2.5, buffer concentration: 100 mM, pH of aqueous solution: 11. 3: Buffer pH = 2.6, buffer concentration: 102 mM, pH of aqueous solution: 11.5. 4: Applied voltage: 24.5 kV, temperature: 14.5 °C, volume of chloroform: 116 μL. 5: Applied voltage: 25 kV, temperature: 15 °C, volume of chloroform: 120 μL. 6: Applied voltage: 25.5 kV, temperature: 15.5 °C, volume of chloroform: 124 μL.

LLOQ concentrations were considered to evaluate the effect of interfering compounds on extraction efficiency of verapamil enantiomers. The proposed method offers the specific and selective analysis of verapamil enantiomers in human plasma. Fig. 7 shows some electropherograms collected using the proposed DLLME-FASI-CE procedure in the presence of racemic verapamil (0.05 μg mL^{-1}) spiked with different drugs. The verapamil enantiomers are well separated from interferents, and the calculated results are within ±20% of the nominal values.

Stability

To monitor the influence of the time interval between sample collection and sample analysis, two procedures were carried out. Short-term temperature stability measurement tests were performed on three concentration levels after thawing at room temperature for 12 h.

Freeze–thaw stability was determined by freezing three levels of QC samples for 24 h and then thawing at room temperature. According to FDA guidelines, the samples were considered sufficiently stable as the accuracy was within 80–120%, and the precision was <15%. The results are summarized in Table 7.

Robustness

To measure the susceptibility of the proposed method to minor changes in analytical conditions during routine analysis, such as small changes in pH, BGE composition, *etc.*, the effects of the following changes in separation and microextraction conditions were determined: variations in sample solution pH by ±0.5 pH units; variations in BGE concentration of ±2 mM; variations in BGE solution pH of ±0.1 pH units; applied voltage; temperature; and extraction solvent volume. Under the changed conditions, plasma samples with verapamil concentrations of 150 ng mL^{-1} were subjected to the DLLME-FASI-CE-UV procedure. The relative recoveries varied from 85 to 106%. The obtained results (see Table 8) demonstrate that small changes to the test conditions had no significant effect on the analysis results. In all cases, resolution remained constant ($R_s = 3.8$).

Conclusion

The described method offered enough sensitivity for monitoring verapamil enantiomers in plasma samples using a universal UV detector. The loss in sensitivity due to the path length and small volume of injected sample in CE is partly

compensated by online sample preconcentration. Stacking mode is easily performed on a CE instrument by manipulating the sample matrix and running a program, without any further modification. This is an advantage for routine analyses whereas providing more sensitive detectors such as mass or laser induced fluorescence is not affordable for most analytical laboratories.

Compared to the chromatography methods described in Table 2, the present method was simpler and faster to perform, as neither time-consuming pre-separation nor expensive chiral columns was needed. In addition, the proposed method overcomes the disadvantages of former methods such as laborious sample preparation steps and the use of large amounts of organic solvents. Compared with a previously reported CE-based method,[16] the present method exhibits a better LLOQ (25 ng mL^{-1}). This is the first report of a DLLME procedure prior to CE for the enantioseparation of verapamil in biological samples. The method is applicable for therapeutic drug monitoring studies and is validated according to FDA guidance.

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