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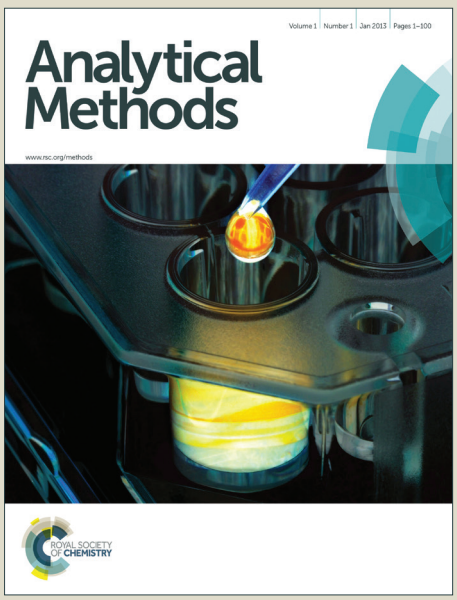

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Capillary electrophoresis with UV detection, on-line stacking and off-line dispersive liquid-liquid microextraction for determination of verapamil enantiomers in plasma

Samin Hamidi[a], Abolghasem Jouyban[b,c*]

[a]Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz 51664, Iran

[b]Drug Applied Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz 51664, Iran

[c]Kimia Idea Pardaz Azarbayjan Science Based Company, Tabriz University of Medical Sciences, Tabriz 51664, Iran

* Corresponding author. Email: ajouyban@hotmail.com.

Tel: +98 41 33379323

Fax: +98 41 33363231

Abstract

A rapid, convenient, sensitive and reliable dispersive liquid–liquid microextraction (DLLME) method was coupled with field-amplified sample injection (FASI) in capillary electrophoresis with diode array detector DAD for 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 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 guideline. This is the first work showing the possibility of the use of DLLME and on-line sample pre-concentration techniques for analysis of verapamil enantiomers in plasma.

Keywords

Capillary electrophoresis; Field-amplified sample injection; Dispersive liquid–liquid microextraction; Verapamil enantiomers

Introduction

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

Some receptors and enzymes present stereo-selectivity to different enantiomers of drugs, therefore two isomers of verapamil distinguish in their bioavailability,[4] pharmacokinetic[5] as well as pharmacodynamic effects.[6] (S)-enantiomer being more active (about 20 times) than (R)-enantiomer.[7] So, the need for a rapid, low cost and efficient analytical enantioseparation method for monitoring of verapamil enantiomres gain much attention and a number of analytical methods have been reported for determination of its enantiomers in bio-fluids. Table 2 listed 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 in the 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 regarding 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 and UV detector is routinely established in most of the laboratories. One of the most important drawbacks of CE-UV is its short optical path length and small volume of the injected sample which cause a loss in

detection sensitivity. To deal with this limitation, some techniques have been emerged such as using extended light path capillaries or providing the more sensitive detectors.

Additionally, in-column pre-concentration techniques were established as efficient ways for enhancing the detection limit in 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 lays on mismatching the ionic strength of sample matrix and BGE, resulting difference in conductivity and concentrates the analyte in narrow sharp zone. This approach can decrease detection limit of drug monitoring in biological samples without special modification of the instrument. In order to provide the necessary conductivity difference between sample and BGE, sample is usually prepared in a low-conductivity matrix.

The determination of analytes in biological samples needs to employ a selective, sensitive, precise and accurate preparation method. Due to the high protein content of plasma and the subsequent clogging risk for the capillary column, direct injection was not recommended in CE. Additionally, as described above, preparation of sample in low conductivity matrix enhances the sensitivity of FASI.

Traditional sample preparation methods such as liquid-liquid extraction (LLE) usually suffer from the disadvantages such as time-consuming set-up, consumption of large quantities of toxic solvents and evaporation of solvent (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 solvents consumption) as well as simplification of sample preparation step. Dispersive liquid-liquid microextraction (DLLME) is one of the latest modes of liquid phase extraction which is based on a ternary

phase solvent system. Contact surface between sample and extraction solvent in DLLME is enlarged by rapid injection of a mixture of extraction and dispersive solvents to sample solution.[24] It has some merits including; fast operation, no need to large amounts of hazardous solvents, low cost and easy coupled 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 affecting on DLLME procedure. Additionally, the applicability of FASI is evaluated. Finally, the DLLME-FASI-CE method was validated for the analysis of verapmil enantiomers in plasma according to FDA guideline.

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 grades. De-ionized (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 1000 μg/mL solution and the desired concentrations of drug were prepared by dilution of stock solution with methanol. Buffer was composed of 100 mM phosphoric acid adjusted to pH 2.5 with TEA and was

prepared fresh daily. The BGE used for 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. Also verapamil spiked plasma samples were 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 uncoated fused-silica capillary 50 μm i.d. and 50 cm total length (41.5 cm effective length) and were purchased from Agilent Technology (Waldbronn, Germany). A vortex from Labtron Company (Tehran, Iran) was used in sample preparation. Sigma centrifuge (Osterode, Germany) was used in protein precipitation step and Hettich centrifuge (Tuttlingen, Germany) was used for sedimentation of the extraction solvent in sample preparation. pH adjustments were made by a Meterohm® pH meter (Herisau, Switzerland). Alex machine (Istanbul, Turkey) was used for ultrasonic performance.

Electrophoretic procedure

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 the BGE (5 min). In order to FASI performance, samples were prepared in 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 0.20-μm pore size PTFE filter (Chromafil, Germany). The samples are introduced into the capillary using electrokinetic injection at 15

kV for 30 s. A short plug of DI water (50 mbar for 1 s) was loaded before sample injection. Capillary was thermostated at 15 ˚C. Applying voltage was 25 kV and on-line UV detection was set on 200 nm based on maximum sensitivity.

Sample preparation and dispersive liquid–liquid microextraction

400 µL drug-free plasma placed at room temperature for 20 min to thaw and spiked with 0.5 µg/mL of the racemic verapamil. 800 µL of acetone was added to the microtube containing spiked plasma and vortexed for 40 s followed by centrifuging for 5 min in 12470 × g to precipitate the proteins.

The yellowish supernatant (1 mL) was placed in a 10-mL glass conical button tube, then was diluted with 9 mL of aqueous solution (pH 11.0 adjusted with NaOH 1.0 M) to ensure that the analyte is in its neutral form. DLLME procedure was performed by quick injecting of 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 in consequence of dispersing chloroform within the solution. The targeted analyte was extracted into tiny droplets and collected using centrifugation in 2307 × g for 5 min. The organic phase was withdrawn with a pipettor after discarding the upper aqueous solution and was 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 the

selector, the eneantiomers were not resolved, the best resolution is achieved at 0.8 % of CM-β-CD and at higher concentrations of selector the resolution was decreased (Figure 1).

Enantioseparation shows significant dependency on pH especially because of pH effect on electro-osmotic flow (EOF). Low pH gives the slow EOF and the analyte migrate 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, employing phosphoric acid–TEA buffer. The ideal separation occurred at pH 2.5. At this pH, CM-β-CD exists as neutral form (pKa 4.36) while verapamil is mostly charged.

Concentration of the buffer was tested (50, 75, 100 and 120 mM) 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 was occurred.

The effect of temperature on the separation could be linked to a number of parameters including viscosity of BGE, extent of interaction between analyte and selector. The effect of temperature was investigated in the range of 15-20 ˚C. With decreasing the temperature to 15 ˚C, the migration time is increased whereas the resolution is improved. So, the instrument was set at 15 ˚C.

To evaluate the influence of organic solvent on the resolution, different amounts of methanol (5-35 % (v/v)) were added to BGE. Increasing the methanol percentage up to 30 % caused to resolution improvement. Increasing 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 get acceptable levels of sensitivity, FASI was used as an on-line sample preconcentration. FASI method is performed by preparing the sample in less conducting

media than BGE. When the voltage is applied, the sample zone migrates faster until it reaches to the zone with high conductivity (lower electric field strength) and causing to concentrate the analytes. Several parameters need to be optimized for the proper execution of FASI including the composition of the sample matrix, injection time, voltage and the presence/absence of high-resistivity plug before sample introducing.

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) 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 it can be seen from Figure 2A, the signal for the analytes significantly increased in 50% ACN.

Performing FASI 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. Exceeding the time of injection over 1 s, caused to disrupting the resolution. So, water plug was injected at 50 mbar for 1 s helps to slightly increasing in signal intensity.

Injection time (15-30 s) and injection voltage (10-20 kV) were checked. Regarding the values of signal as well as resolution, voltage was selected at 15 kV. As shown in Figure 2B the best amplification of the signal was observed in the electrokinetic injection at 15 kV for 30 s. Injection times longer than 30 s caused to 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 injection of water plug at 50 mbar for 1 s

while temperature was kept at 15 ˚C and the applied voltage was 25 kV. Samples were prepared in the mixture of 100 µL ACN/water (1:1 (v/v)).

Investigation of protein precipitation step

Protein precipitation processing is necessary for fast clean-up as well as protein- drug binding cleavage in plasma.[27] 400 µL of plasma samples were spiked by 0.5 µg/mL of racemic verapamil in a 2-mL polypropylene microtube and left for 20 min at room temperature. Simplification of complex plasma matrix with organic solvents was tested in the presence of various solvents including: acetone, ACN and mixture of acetone and ACN separately with 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)), respectively and solutions vortexed for 40 s, then centrifuged for 5 min at 12470 × g. They subjected to described DLLME procedure, and then analyzed using CE. Corresponding data for investigation the appropriate precipitant are shown in Table 3.

According to high signal obtained on detector, protein precipitation was performed by addition of 800 µL of acetone to 400 µL of plasma.

Investigation of DLLME performance

According to high signal obtained on detector, protein precipitation was performed by addition of 800 µL of acetone to 400 µL of plasma.

Optimization of extraction solvent: type and volume

To achieve an efficient extraction, the extraction solvent should possess some requirements. 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 column (such as halogenated

solvents) as well as concerning the necessity of extract concentration, ease of evaporation of solvent should be regarded. Chlorinated solvents such as $CHCl_3$, CCl_4 and CH_2Cl_2 are good choices. By applying 100 µL of each solvent to DLLME procedure, effect of extraction solvent was evaluated. No sediment phase was observed when 100 µL of CH_2Cl_2 was applied as an extraction solvent which probably was attributed to its higher solubility in aqueous solution. Corresponding signals using CCl_4 are 7.5±0.1 and 7.4±0.1 for the first and second enantiomers, respectively. By applying $CHCl_3$, the obtained signals were 7.9±0.1 and 7.9±0.1 for each verapamil enantiomer. Consequently, the best signal was achieved by $CHCl_3$ and was employed in the subsequent studies.

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

Optimization of method to form emulsion

Generation of tiny droplets for increasing the contact area between extraction solvent and aqueous phase is the key step in DLLME. The cloudy solution could be formed using dispersive-solvent-free manner, *e.g.* using ultrasound,[28] vortex[29] or air.[30] The purpose is promoting the turbidity which caused to an increase in the contact surface area which results in mass transfer of target analyte from sample solution to organic phase. In practice, formation of cloudy state was followed by means of several methods such as sonication, vortex and air instead of using dispersive solvent. 120 µL chloroform (as an extraction solvent) was introduced in the conical tube containing sample solution (was prepared as mentioned in sample preparation section). The tube was immersed into ultrasonic water bath

and extraction was performed for 10 min at 25 ˚C. In another set-up, In order 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 extraction procedure, the sample mixture was rapidly sucked into a 5 mL syringe and then was injected into the tube (five times) via syringe needle.

After centrifuging the obtained cloudy solutions, white floccus phase settled down on the interface of the two phases which made difficulties to collect the organic phase.

Compared to ultrasonic liquid-liquid microextraction set-up, DLLME approach requires simple laboratory equipment and was performed in a couple of minute. Most of the reported dispersive-solvent-manners, such as those tested in the present work, are dealing with aqueous solutions, whereas samples with biological origin exhibit completely different behavior. Utilizing the other dispersion methods instead of dispersive solvent in biological samples is seldom reported. In complex matrices, recovery the organic phase after dispersion into the sample with the aid of any dispersion agent, is a challenging issue. Therefore, the superiority of the use of each technique instead of dispersive solvent for creating the infinite contact between analyte and extraction solvent should be regarded considering the extent of organic phase recovery.

At the present work, in addition to longer time, these methods resulted in lower efficiencies. So, in this work using the dispersive solvent was adapted for next experiments.

Optimization of dispersive solvent: type and volume

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

Sample solution (consisted of diluted plasma + acetone (added as protein precipitant) was treated with mixture of 120 µL chloroform and different dispersive solvents including methanol, acetone, ACN and THF and was analyzed as described previously in DLLME performance section. The two phase system other than methanol was formed well for all the candidates. Acetone forms good dispersion solution and give high signal (Figure 3B), therefore acetone was selected as dispersive solvent for next analysis. The volume of dispersive solvent is an important parameter that shows a crucial effect on extraction efficiency. Different volumes of acetone (300, 500, 700 and 900 µL) were investigated. As can be observed in Figure 3C, the signal of analytes increased up to 500 µL and then decreased with the increased volumes of acetone. 0.5 mL of acetone was chosen was chosen as the appropriate volume of dispersive solvent.

Optimization of centrifugation time and rate

DLLME finishes with centrifugation in order to recover organic phase droplets containing target analyte at the bottom of the tube. To monitor this subject, rate and time of centrifuge were studied in the ranges of 1153–2883 × g and 3–10 min, respectively. These parameters show less effectiveness (Figure 4) and finally 2307 × g for 5 min were chosen as optimum values.

Sample dilution effect

Due to the complexity of plasma media, matrix effect is suspected. Therefore, the effect of sample volume on extraction efficiency was investigated in the volumes of 5 to 9 mL. 1 mL of plasma after spiking and protein precipitation was used in all studied volumes. Analytical response increased upon 1:9 (sample: aqueous phase (v/v)) dilution. Results of optimization were shown in Figure 5.

Method validation

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 by spiked plasma samples and were treated with racemic verapamil following optimized DLLME performance in a specific concentration range of 50-700 ng/mL. The linearity of 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. LLOQs cover the expected value for therapeutic drug monitoring purpose.

Precision, accuracy and recovery

Precision was used for comparing the uncertainty between different measurements and expressed as %RSD. The RSD% of each level were 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:

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

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 listed in Table 5. The results demonstrated that the values were within the acceptable range and the method was accurate and precise.

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

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

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 for recovery calculations are present in Table 6.

Specificity and selectivity

The specificity of method was determined by introducing the blank sample which was prepared as the same way explaining previously. The method is specific as no interfering peaks are present in drug-free plasma. Figure 6 displays the electropherograms of drug-free plasma and spiked plasma under optimal DLLME-FASI-CE-UV. In order to follow the selectivity of the assay, plasma samples were 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 and injected to CE using the same condition at concentrations of 0.5 µg/mL

for each analyte and analyzed according to the proposed procedure. At the migration time of verapamil enantiomers, no overlap peak was found. The LLOQs concentrations have been considered as evidence. The proposed method offers the specific and selective analysis of verapamil enantiomers in human plasma. Figure 7 shows the some electropherograms in the presence of spiked racemic verapamil (0.05 µg/mL) with different drugs under DLLME-FASI-CE performance. The verapamil enantiomers are well arisen from interferes and the calculated results are within ±20% nominal value.

Stability

To monitor the influence of the time intervals between sample collection and sample analysis, two procedures were carried out. Short-term temperature stability measurement tests performed on three levels of concentrations that thawed at room temperature and left for 12 h. Freez-thaw stability was carried out by freezing three levels of QC samples for 24 h, followed by thawing at room temperature. According to FDA guideline, the samples were considered stable enough concerning the accuracy was within 80–120% and precision was <15%. Results are summarized in Table 7.

Robustness

To measure the susceptibility of the proposed method to minor changes in analytical conditions during routine analysis like small changes of pH values, BGE composition, sample solution pH etc. The effects of the following changes in separation and microextraction conditions were determined: sample solution pH variations by ±0.5 pH units, BGE concentration and its pH adjusted by ±2 mM and ±0.1 pH units, respectively, applied voltage, temperature and extraction solvent volume. Under the changed conditions, plasma samples with the verapamil concentration of 150 ng/mL 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 applied in test conditions had no significant effect on the analysis results. In all cases resolution was remained constant (R_s=3.8).

Conclusion

Described method offered enough sensitivity for monitoring verapamil enentiomers in plasma samples using universal UV detector. Loss in sensitivity from the path length and little volume of injected sample in CE is partly compensated by online sample preconcentration. Stacking is easily coupled with CE by manipulation in sample matrix without interfere in the time of performance or in any further modification of commercially available CE instrument, whereas providing more sensitive detectors such as mass or laser induced fluorescence are not affordable for most of biomedical analysis laboratories.

In comparison to chromatography methods which are indicated in Table 2, the present method was simpler and faster to perform, as neither time-consuming pre-separation nor expensive chiral columns were needed. In addition, proposed method overcomes disadvantages of former methods such as laborious sample preparation step and use of large amounts of organic solvents. Present method compared with previously reported CE-based method[16] shows rather better LLOQs (25 ng/mL). This is the first report on DLLME procedure prior to CE for 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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Tables

Table 1: Molecular structure and physicochemical parameters of verapamil.

Table 2: Some previous studies for determination of verapamil enantiomers in biological fluids.

Table 3: Details of protein precipitant optimization on 0.5 µg/mL spiked verapamil sample.
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Table 5: Assay precision and accuracy of QC samples spiked with verapamil.

Table 6: Recoveries for extraction and analysis of verapamil in spiked plasma samples with DLLME-FASI-CE-UV.

Table 7: Evaluation of method stability for extraction and determination of verapamil enantiomers in spiked human plasma.

Table 8: Results of the evaluation of method robustness in six different levels.

Figures

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

Figure 2: Optimization of the injection parameters for FASI. 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% of 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 of racemic drug injected at 15 kV for 30 s and (B) sample injection time; sample solution: 0.5 µg/mL of racemic drug injected at 15 kV after preliminary pressure injection of water (50 mbar for 1 s) and the error bars indicate the SD (n=3).

BGE: Background electrolyte; TEA: Triethanolamine; CM-β-CD: Carboxy methyl beta cyclodexterin; FASI: Field-amplified sample injection.

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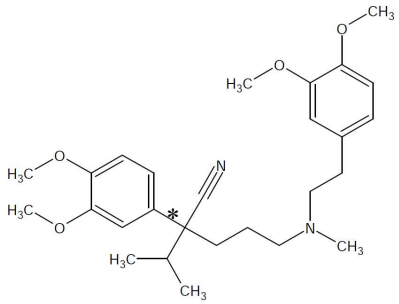
Table 1: Molecular structure and physicochemical parameters of verapamil.

	Therapeutic range (ng/mL)	50-250[2]
	Log P	3.8[3]
	pKa	8.9[3]

*Indicates the chiral center.

Table 2: Some previous studies for determination of verapamil enantiomers in biological fluids.

Method	Sample type/ size	Sample preparation	Linear range single enantiomer (ng/mL)	LOQ single enantiomer (ng/mL)	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/ 0.5mL	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	250-10000	200	Precision, accuracy of intra- and inter-day analysis, selectivity and recovery	[16]

LLE: Liquid-liquid extraction, TM-β-CD: Trimethyl-β-cyclodextrin

Table 3: Details of protein precipitant optimization on 0.5 µg/mL 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

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

Analytes	Calibration curve	R^2	Linear range (ng/mL)	LOD (ng/mL)	LLOQ (ng/mL)
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.

Analytes	Nominal concentration (ng/mL)	Intra-day RSD%; n=5[a]	Inter- day RSD%; n=5[b]	Accuracy (RE%)[c]
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).

Table 6: Recoveries for extraction and analysis of verapamil in spiked plasma samples with DLLME-FASI-CE-UV.

Analytes	Nominal concentration (ng/mL; n = 5)	Mean found concentration (ng/mL)	Mean recovery (%)
Enantiomer 1	25	28	115
	150	139	92
	250	249	99
Enantiomer 2	25	27	108
	150	143	95
	250	249	99

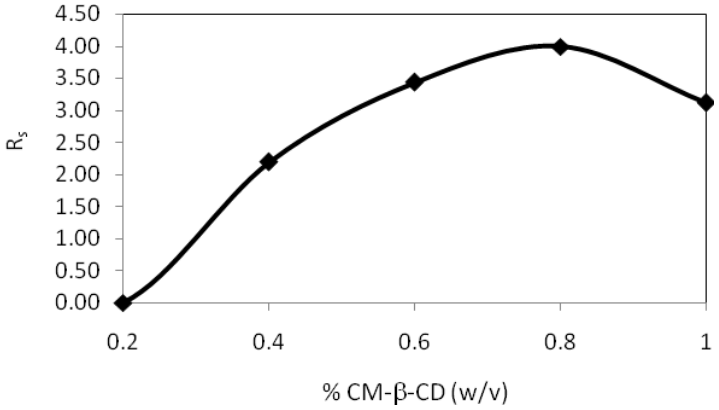
Table 7: Evaluation of method stability for extraction and determination of verapamil enantiomers in QC human plasma.

Analytes	Nominal concentration (ng/mL; n=3)	Freeze-thaw stability		Room temperature stability	
		Mean found concentration (ng/mL)	Accuracy (%RE)	Mean found concentration (ng/mL)	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 in six different levels.

Analytes	Level	Nominal concentration (ng/mL; n=3)	Mean found concentration (ng/mL)	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

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, Temperature: 14.5 ˚C, Volume of chloroform: 116 µL.
5: Applied voltage: 25, Temperature: 15 ˚C, Volume of chloroform: 120 µL.
6: Applied voltage: 25.5, Temperature: 15.5 ˚C, Volume of chloroform: 124 µL.



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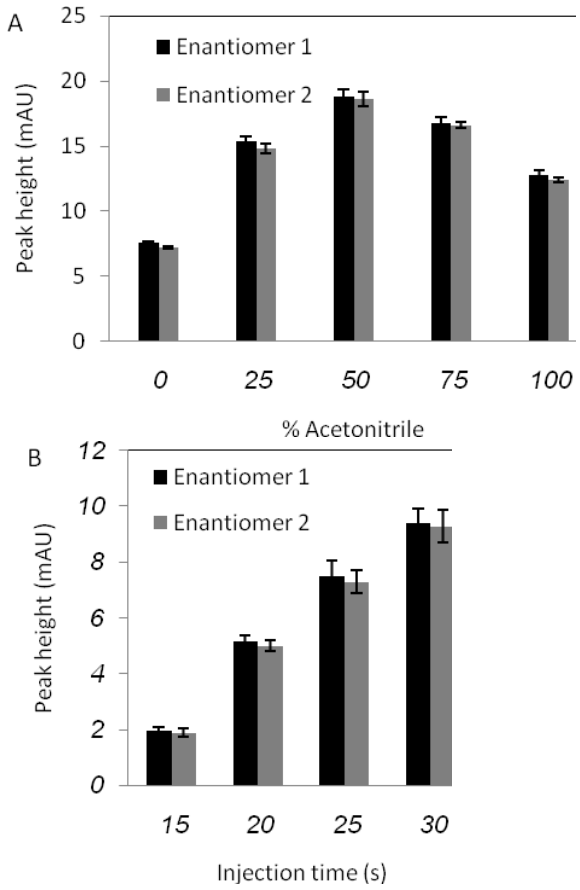

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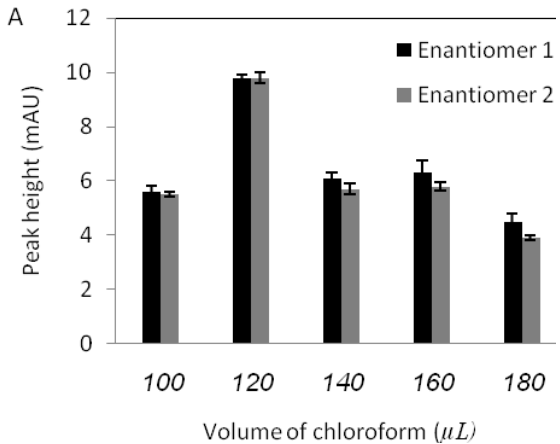

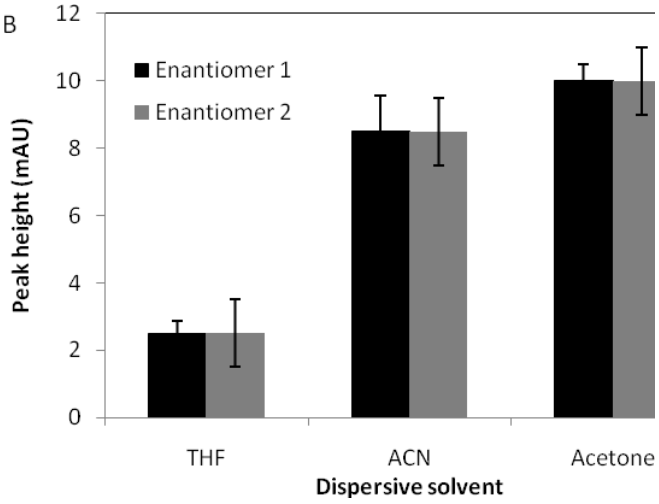

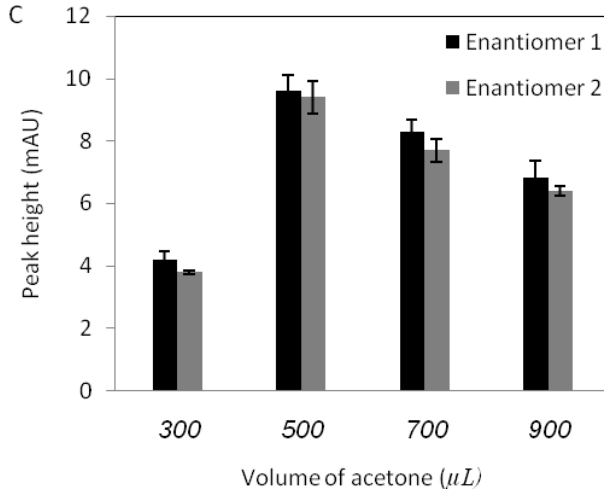

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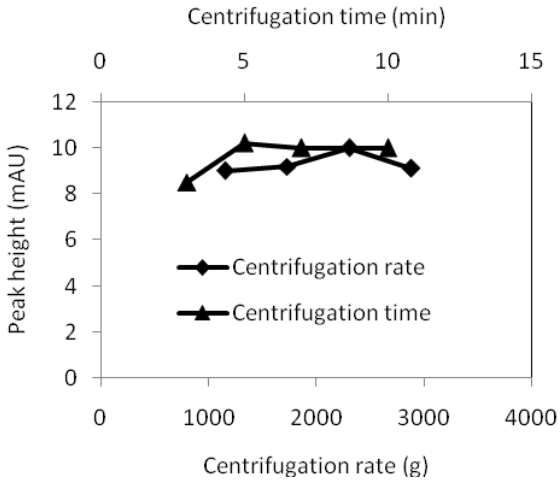

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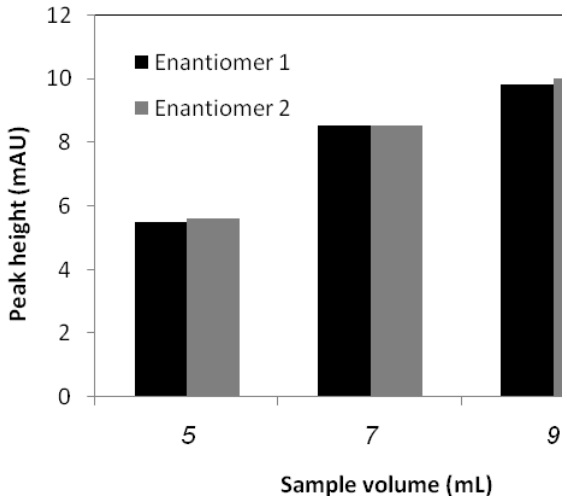

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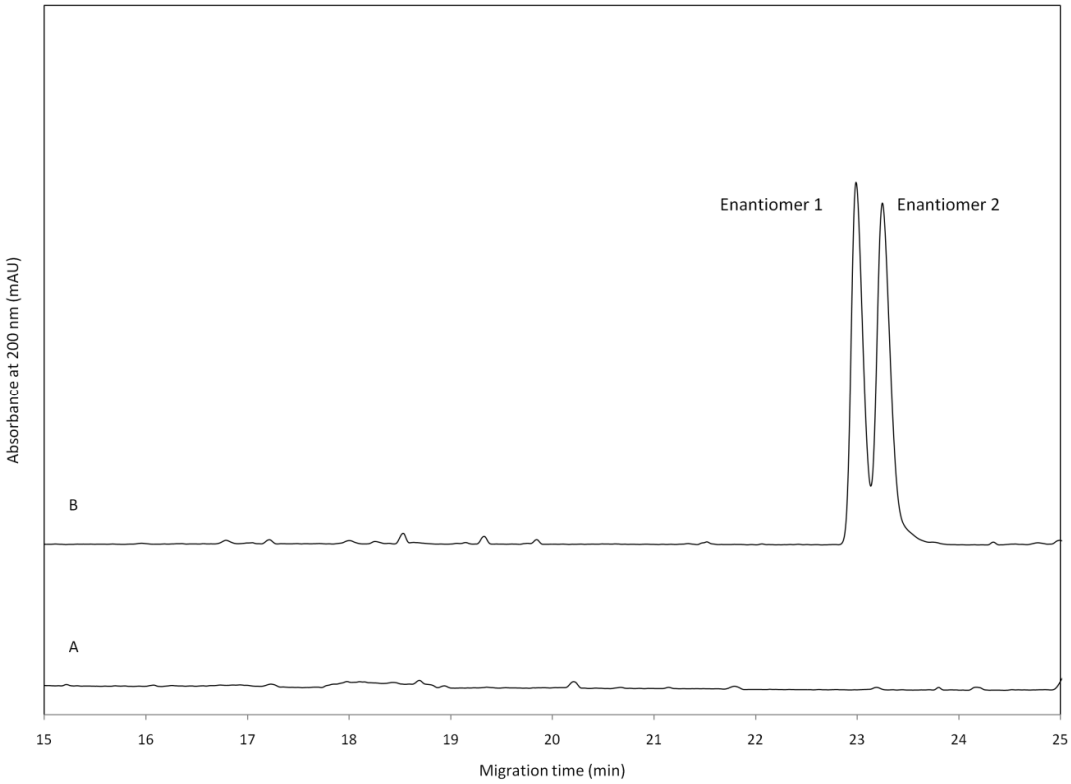

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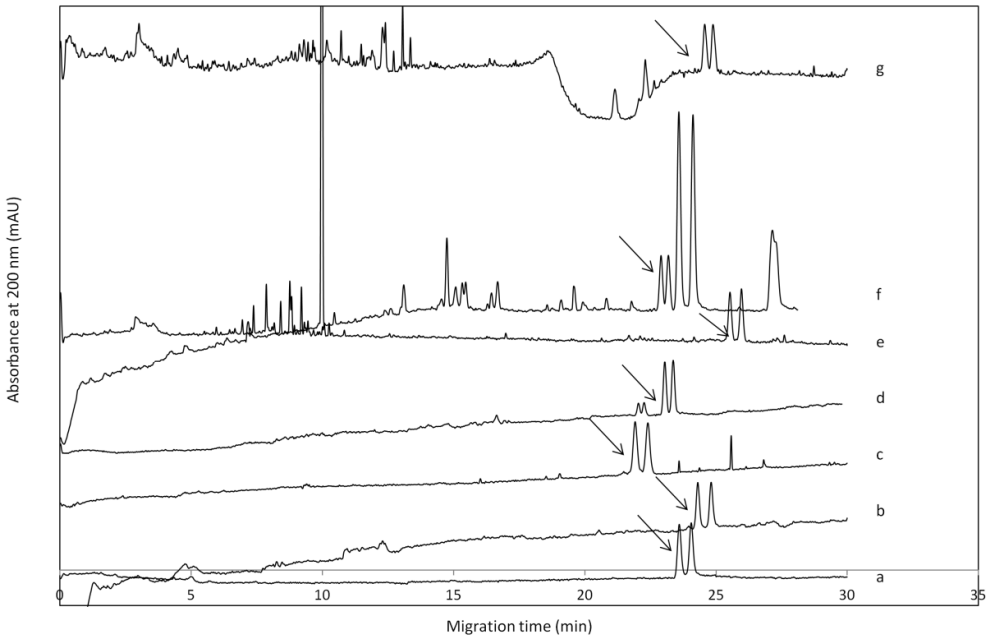

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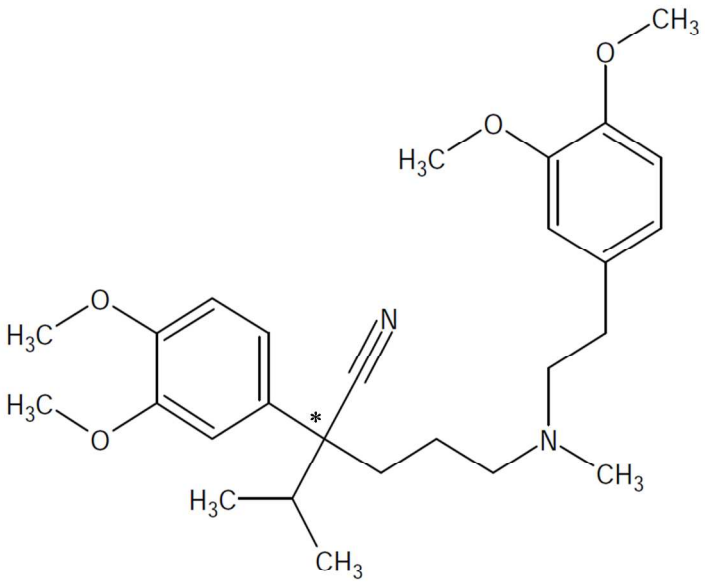

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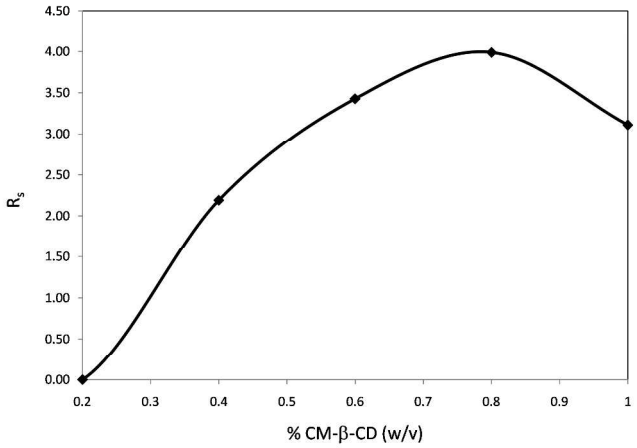

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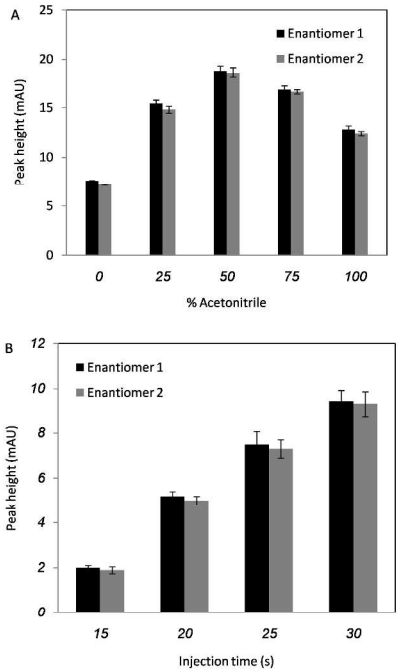

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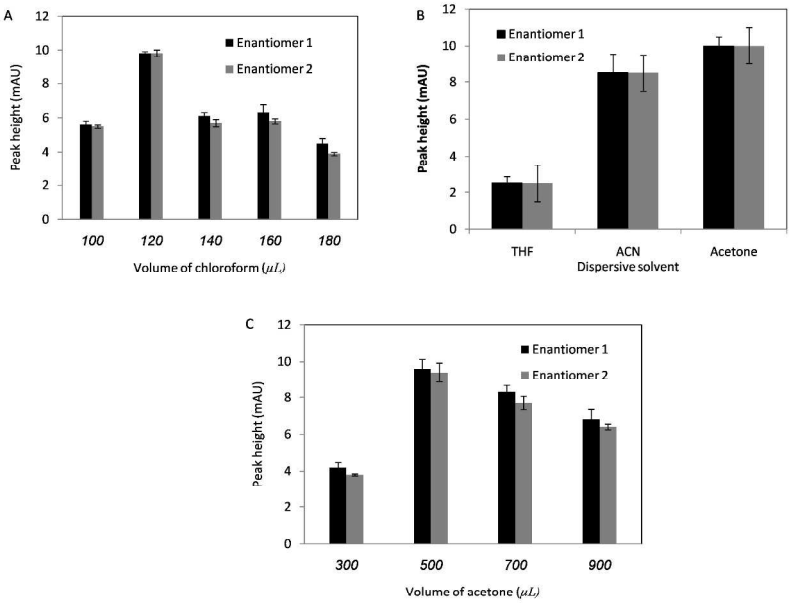

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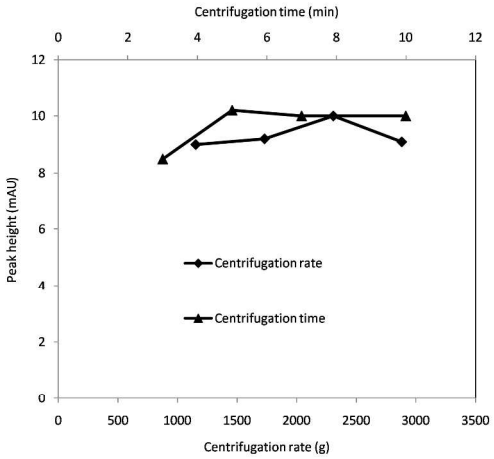

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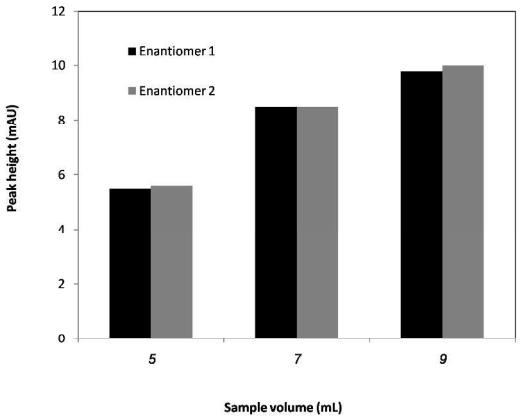

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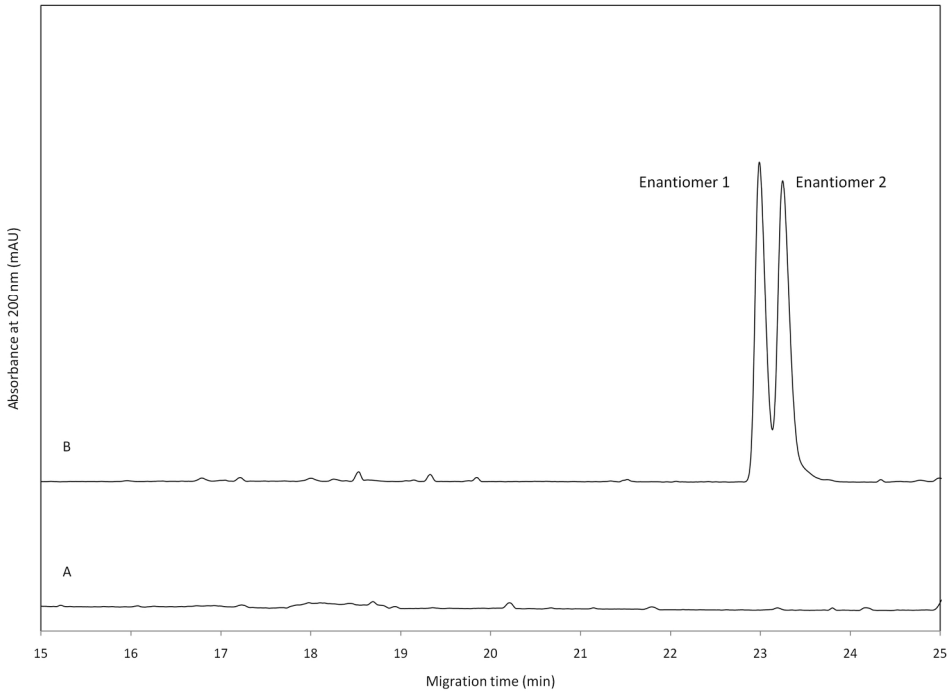

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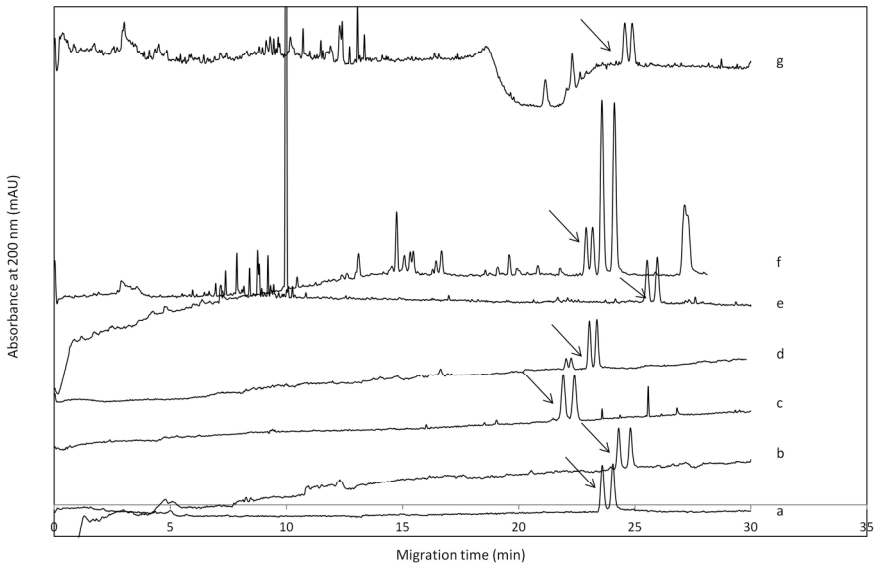

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