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DETERMINATION OF CELECOXIB IN HUMAN PLASMA BY EFFERVESCENCE-ASSISTED DISPERSIVE LIQUID–LIQUID MICROEXTRACTION AND HPLC/UV

(Penentuan Celecoxib dalam Plasma Manusia dengan Bantuan Buih Berbuak Pengekstrakan Mikro Penyebaran Cecair-ke-Cecair dan HPLC/UV)

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Abstract

In this study, effervescence-assisted dispersive liquid–liquid microextraction was utilized for rapid, simple, and inexpensive microextraction of celecoxib followed by HPLC/UV. In the proposed method, carbon dioxide microbubbles were produced by the reaction between oxalic acid and sodium bicarbonate. Various factors such as type and volume of extraction solvent, pH of the sample solution, content of the acidic and basic promoters, and type and volume of auxiliary solvent were optimized. Under optimum conditions, 60 µL chloroform (as an extraction solvent) was added to 2 mL methanol and 0.4g oxalic acid. The mixture was added to the test tube containing 0.8g sodium bicarbonate and 10 mL of sample solution (pH 6). The presented method offered wide linear dynamic range of 0.5-1000 µg L^{-1} with satisfactory extraction recovery of 94.4%, high pre-concentration factor of 206, and normal RSD% (2.15, n=3). Finally, the proposed method was successfully applied for the analysis of celecoxib in plasma samples.

Keywords: effervescence-assisted liquid-microextraction, celecoxib, plasma, acid-base reaction, carbon dioxide microbubbles

Abstrak

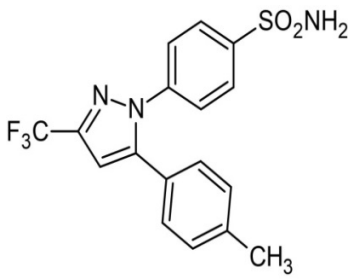
Dalam kajian ini, pengekstrakan mikro cecair-ke-cecair yang dibantu buih berbuak telah digunakan untuk pengekstrakan celecoxib yang cepat, mudah, dan murah diikuti oleh HPLC/UV. Dalam kaedah yang dicadangkan, buih mikro karbon dioksida dihasilkan oleh tindak balas antara asid oksalik dan natrium bikarbonat. Pelbagai faktor seperti jenis dan isipadu pelarut pengekstrakan, pH larutan sampel, kandungan penggalak asid dan bes, dan jenis dan isipadu pelarut bantu telah dioptimumkan. Di bawah keadaan optimum, 60 µL kloroform (sebagai pelarut pengekstrakan) ditambahkan ke dalam 2 mL metanol dan 0.4g asid oksalik. Campuran tersebut ditambahkan ke dalam tabung uji yang mengandungi 0.8g natrium bikarbonat dan 10 mL larutan sampel (pH 6). Kaedah yang dikemukakan menawarkan julat dinamik linear yang luas dari 0.5-1000 µg L^{-1} dengan pulihkan pengekstrakan yang memuaskan sebanyak 94.4%, faktor pra-pemekatan tinggi sebanyak 206, dan RSD% normal (2.15, n=3). Akhirnya, kaedah yang dicadangkan telah berjaya digunakan untuk analisis celecoxib dalam sampel plasma.

Kata kunci: pengekstrakan cecair-mikro yang dibantu buih berbuak, celecoxib, plasma, tindak balas asid-bes, buih mikro karbon dioksida

Introduction

Non-steroidal anti-inflammatory drug (NSAID), celecoxib (CEL) has been extensively prescribed for the treatment of inflammation and pain in osteoarthritis, rheumatoid arthritis, and painful menstruation [1]. Furthermore, the therapeutic effect of this drug on renal and pancreatic functions, as well as its chemo-preventive effect on various cancer types have been recently investigated and reported [2]. In spite of the excellent therapeutic effect of CEL, its high dosage can enhance the risk of heart attack. Some recent reports have also mentioned the distribution of CEL into human breast milk [3, 4]. In this context, the development of an efficient technique for the measurement of CEL concentration in biological matrices can contribute to optimizing the CEL dosage based on medical strategies and therapeutics for serious intoxication. The most common analytical method for measuring the CEL in bio-fluid samples is based on high-performance liquid chromatography (HPLC) [5-8]. The quality of analysis usually decreases due to the complexity of bio-fluids matrices and trace concentration of CEL. Hence, the development of a sample preparation technique to reduce the matrix interference and enrich the trace values of CEL is the main challenge.

The structure and corresponding log P value (octanol–water partition coefficient) and dissociation constant value (pKa value) of CEL are presented in Figure 1.



Log p= 3.5 pKa= 11.1

Figure 1. Chemical structure, Log p and pKa of Celecoxib

Extraction of trace contents of chemical compounds that can be analyzed by analytical instruments has found increasing popularity among the scientists. This extraction should be able to concentrate analytes and also sample cleanup contemporaneously. This process is called sample preparation [9, 10]. Liquid- liquid extraction (LLE) and Solid phase extraction (SPE) are among the earliest sample preparation methods [11, 12]. Scientists have also attempted to minimize the consumption of organic solvents and simplify this type of extraction. Liquid–liquid microextraction (LLME) and Solid phase microextraction (SPME) are really useful in the extraction of the analytes from various matrixes [13-15]. Liquid phase microextraction (LPME) is an interesting and applicable method capable of extracting analytes from an aqueous phase in mL to an organic solvent in μL [16, 17].

Among the conventional solvent-minimized extraction methods, dispersive liquid liquid microextraction (DLLME) is frequently applied due to its outstanding benefits such as fast microextraction process, high enrichment factor, and low consumption of organic reagents as extraction solvents [18-21]. In spite of the excellent performance of this method, there are several disadvantages in the initial version of DLLME method. The low partition coefficient of the target analytes due to use of the organic disperser solvents and inadequate contact surface between the organic extractor and sample solution are the shortcomings of the DLLME method. The use of hazardous chlorinated extractors and toxic disperser solvents increases the operational and environmental pollution risks which it is the other drawback of the mentioned method [22]. In recent years, effervescence-assisted dispersive liquid–liquid microextraction (EADLLME) method has been proposed as a new assisted technique with promising potential for pre-concentration of target analytes without using the disperser solvent [23-27].

The present study is focused on the development of EADLLME technique based on gas-generation reaction which could be an appropriate alternative for organic disperser solvent in DLLME method. In this method, CO_2 microbubbles are formed as disperser by the reaction between acid and base source in the sample solution. The produced CO_2 microbubbles enhance the dispersibility of organic extraction solvent which promotes the transfer of CEL to the organic phase. Also, the gas bubbles provide the large contact surface between CEL and extractant, a high extraction efficiency and rapid extraction equilibrium. The effect of several important factors on the extraction efficiency was investigated by utilizing one-at-a-time process to achieve the optimum micro-extraction conditions.

Finally, the developed EADLLME method was successfully applied to isolate and pre-concentration of CEL in the plasma samples. The analysis of target analyte was accomplished by high-performance liquid chromatography with ultraviolet detection (HPLC/UV). Precise and repeatable data were obtained to find further high pre-concentration factor and extraction recovery.

Materials and Methods

Materials and standard

Celecoxib was supplied from Arasto Pharmaceutical Chemicals Inc (Tehran, Iran). Oxalic acid, sodium bicarbonate, HPLC grade methanol (MeOH), and acetonitrile (ACN) were provided from Merck (Darmstadt, Germany). N-hexane, dichloromethane, carbon tetrachloride, chloroform, methanol and ethanol were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasma samples were obtained from Blood Transfusion Organization (Babol, Iran). Standard stock solution of CEL (500 ppm) was prepared by dissolving 50 mg of reference material into 100 mL of HPLC grade methanol which was refrigerated before use. Working solutions were prepared by diluting the stock solution in water.

Apparatus

HPLC analysis of CEL was carried out using an Alliance HPLC system from waters Company (Milford, USA). The HPLC instrument included a 2695 pump, an online degasser, a UV/vis detector (Model 2487), and a 6-port/3-channel injection valve encompassing a high-pressure manual injection valve (20 µL loop). The detector was adjusted at 252 nm. C_{18} (150 mm × 4.6 mm I.D., 5 µm) MZ-Analysentechnik GmbH HPLC column (Mainz, Germany) was also utilized for chromatographic separation. Empower software was employed for data processing. Isocratic elution was also conducted by a mixture of acetonitrile: water (75:25) at the flow rate of 1mL/min.

Effervescence-assisted dispersive liquid–liquid microextraction procedure

2 mL methanol (auxiliary solvent), 0.4g oxalic acid (acidic promoter), and 60 µL Chloroform (Extraction solvent) were mixed and then added to a conical test tube containing 0.8g sodium bicarbonate(basic promoter) in 10 mL water with the concentration of 1 µg mL^{-1} of CEL (pH 6). The acid-base reaction occurred rapidly upon adding the acidic promoter to the basic promoter solution (Eq. 1) and CO_2 (one of the reaction products) generated bubbles inside the test tube, resulting in a cloudy solution. After one minute, the mixture was centrifuged for 5 min at 5000 rpm and the dispersed droplets of organic phase were gathered at the conical bottom of the test tube. The extract was withdrawn using a micro-syringe and analyzed by HPLC/ UV.

$$2NaHCO_3 + (COOH)_2 \longrightarrow (COONa)_2 + 2CO_2 + 2H_2O \quad (1)$$

Results and Discussion

This work addressed the effects of various parameters such as type and volume of extraction solvent, content of acidic and basic promoters, pH of sample solution and type and volume of auxiliary solvent for the optimization of EADLLME procedure of CEL.

Extraction solvent

The following criteria should be considered in the selection of an extraction solvent for EADLLME: It should be immiscible with water and compatible with chromatographic systems and also have good efficiency for extraction of the analyte. Dichloromethane, chloroform, carbon tetrachloride (higher density than water), and n-hexane (lower density than water) were examined. Among these solvents, chloroform showed the highest Extraction recovery and was used for further studies (Figure 2).

Content of acidic and basic promoter

Four contents of oxalic acid (acidic promoter) and sodium bicarbonate (basic promoter) were examined for optimizing the promoter's content. The molar ratio of $NaHCO_3$: $C_2H_2O_4$ was set to 2:1. The Extraction recovery of celecoxib increased by raising the oxalic acid content to 0.4 g (Figure 3). So 0.4g of oxalic acid and 0.8g of sodium bicarbonate are considered as the optimized amount in the further studies.

Type and volume of auxiliary solvent

An auxiliary solvent dissolves the oxalic acid, promoting the dispersion of the extraction solvent in the aqueous phase. Methanol, ethanol, and acetonitrile were studied for this purpose. Extractions were conducted with oxalic acid (0.4g) and 1 mL methanol, ethanol and acetonitrile. Based on Figure 4, methanol

was the best auxiliary solvent. According to Figure 5, 2 mL methanol led to the highest extraction recovery in the extraction of CEL.

Volume of extracting solvent

Volume of extraction solvent is one of the main parameters that should be optimized. For this purpose, 60, 70, 80, 90, and 100 µL of chloroform were applied. Based on the results, extraction with 60 µL showed the greates extraction recovery, thus, this value was selected for further analyses (Figure 6). Under this volume, no organic solvent sedimented at the bottom of the test tube.

Effect of pH

The pH of the sample solution is another important parameter in the EADLLME method. The effect of this factor was explored on the extraction recoveries of CEL in the ranges of 3.0-8.0. Based on the obtained results (Figure 7), the extraction recovery of CEL decreased by decrementing the pH of sample to 3.0. The target analyte appeared into its ionic form at low pH value which reduced transferring into the organic phase and decreased extraction efficiency. At higher pH levels, the reaction between acid and base is likely to be disrupted, decreasing CO_2 production, followed by a reduction in the dispersion of the organic phase in the sample solution and extraction recovery. Therefore, pH of 6.0 was chosen as the optimum pH for the following experiments.

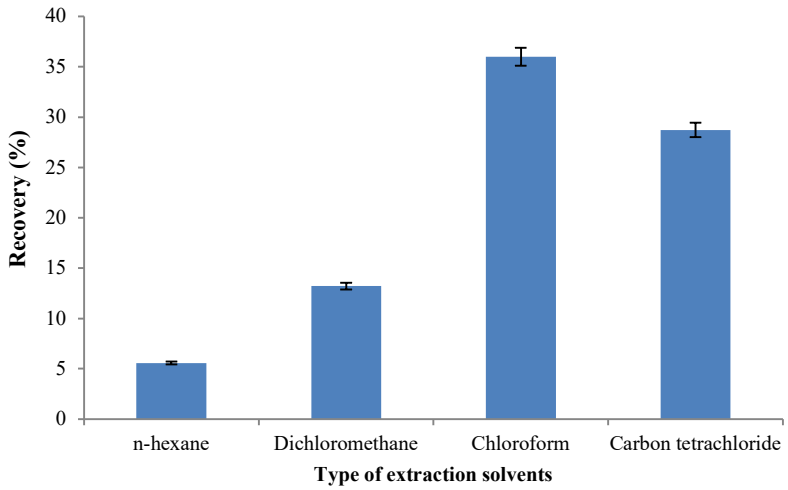


Figure 2. Effect of extraction solvent on the extraction recovery of CEL. Extraction condition: CEL 1µg mL^{-1}; sample volume 10 mL; extraction solvent volume: 100 µL; acidic promoter 0.3g; basic promoter 0.6g; auxiliary solvent; methanol, 1 mL; centrifuging time: 2 min at 4000 rpm. All experiments were done at triplicate and the mean value of the results is reported

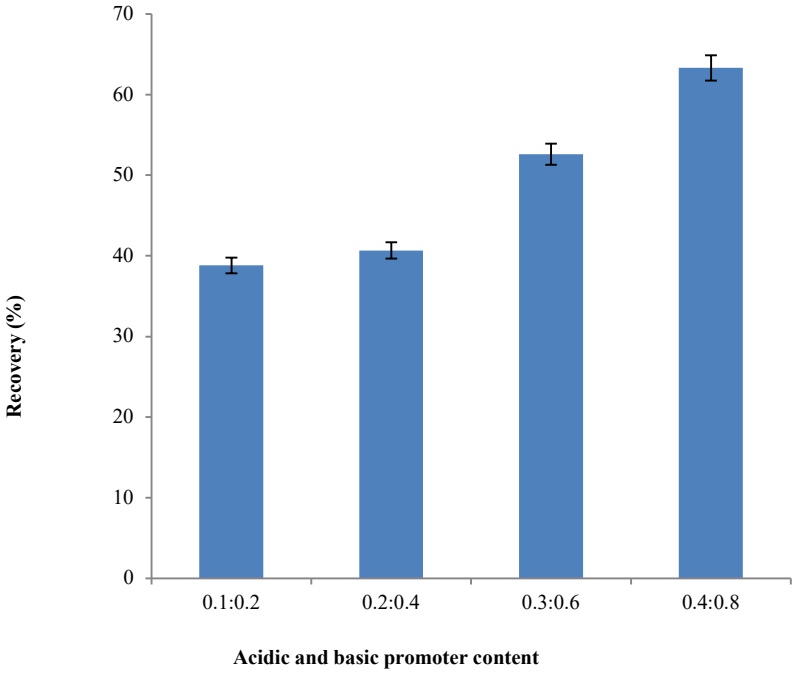


Figure 3. Effect of the content of acidic and basic promoter on extraction recovery. Extraction condition: CEL 1µg mL^{-1}; sample volume 10 mL; extraction solvent: $CHCl_3$, 100 µL; auxiliary solvent; methanol, 1 mL; centrifuging time: 2 min at 4000 rpm. All experiments were done at triplicate and the mean of results is reported

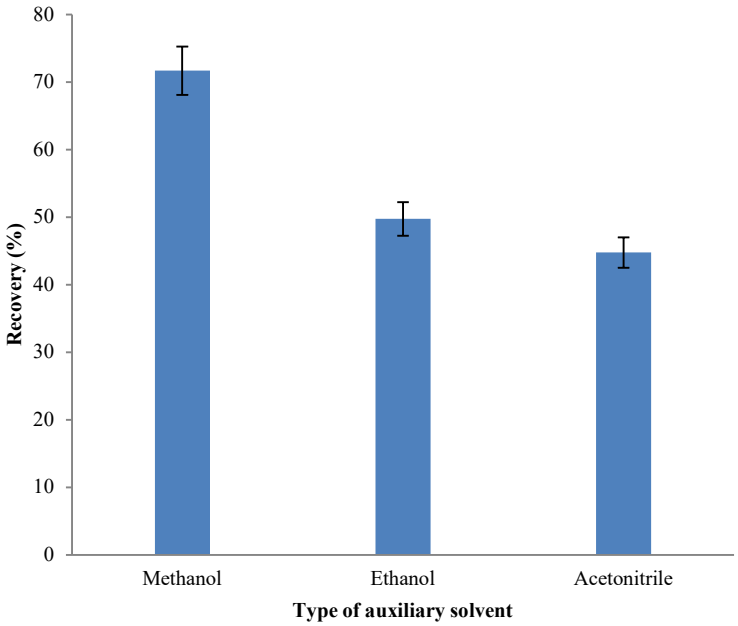


Figure 4. Effect of the type of auxiliary solvent on extraction recovery. Extraction condition: CEL 1 µg mL^{-1}; sample volume: 10 mL; extraction solvent: $CHCl_3$, 100 µL; acidic promoter: 0.4g; basic promoter 0.8 g; volume of auxiliary solvent: 1 mL; centrifuging time: 2 min at 4000 rpm. All experiments were performed in triplicate and the mean values of results are reported

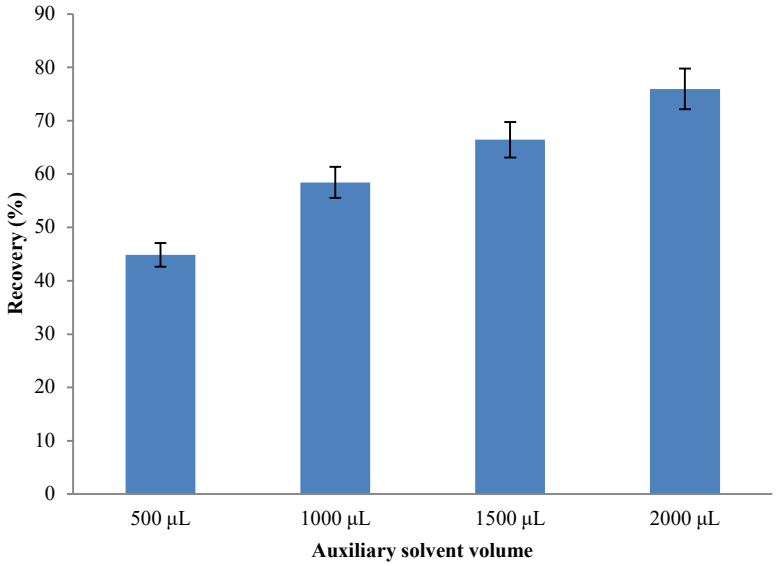


Figure 5. Effect of the volume of auxiliary solvent on extraction recovery. Extraction condition: CEL 1µg mL^{-1}; sample volume: 10 mL; extraction solvent: $CHCl_3$, 100 µL; acidic promoter: 0.4g; basic promoter: 0.8g; auxiliary solvent: methanol; centrifuging time: 2 min at 4000 rpm. All experiments were performed in triplicate and the mean values of results are reported

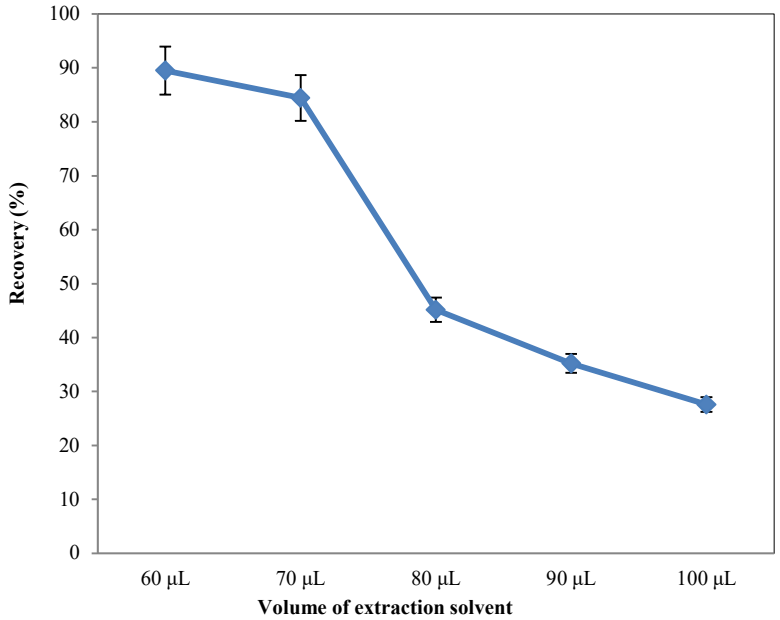


Figure 6. Effect of the volume of extraction solvent on extraction recovery. Extraction condition: CEL 1 µg mL^{-1}; sample volume: 10 mL; extraction solvent: $CHCl_3$; acidic promoter 0.4g; basic promoter 0.8g; auxiliary solvent: methanol, 2 mL; centrifuging time: 2 min at 4000 rpm, pH of sample solution, 6. All experiments were performed in triplicate and the mean value of the results are reported

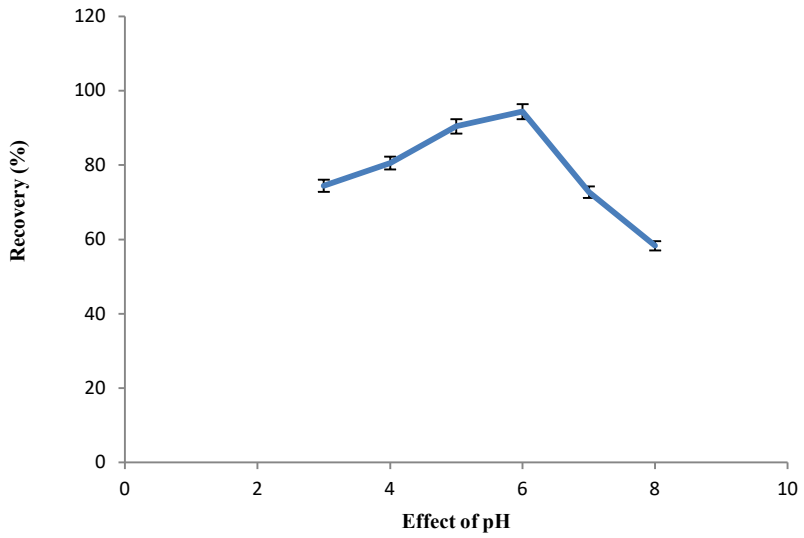


Figure 7. Effect of pH of sample solution on extraction recovery. Extraction condition: CEL 1 µg mL^{-1}; sample volume: 10 mL; acidic promoter: 0.4g; basic promoter: 0.8g; auxiliary solvent: methanol, 2 mL; extraction solvent: $CHCl_3$, 60 µL; centrifuging time: 2 min at 4000 rpm. All experiments were done in triplicate and the mean value of results is reported.

Method performance

The performance of EADLLME was investigated in optimal conditions. The limit of detection (LOD), linear dynamic range (LDR), correlation coefficient (r^2), Enrichment factors (EF), and extraction recovery (ER %) were evaluated which can be calculated using the following equations:

$$\text{LOD} = \frac{3\text{Sb}}{\text{m}} \quad (2)$$

Where S_b is the standard deviation of three replicates in the blank solution and m shows the slope of calibration curve.

Table 1 summarizes the performance of the EADLLME as an approach for extraction of celecoxib.

$$\text{EF} = \frac{Co}{Caq} \quad (3)$$

Where C_o denotes the concentration of the analyte in the organic phase (settled at the conical end of test tube) and C_{aq} represents the concentration of analyte in the aqueous phase.

$$\text{ER}=(\frac{Vo}{Vaq}) \times EF \times 100 \quad (4)$$

Where V_o and V_{aq} are the volume of the organic phase and aqueous phase, respectively [28].

The figures of merits of EADLLME method and other extractions of celecoxib are compared in Table 2.

Table 1. Figures of merit of the presented method

LDR (µg L^{-1})	Correlation Equation	R^2	PF[a]	LOD (µg L^{-1})	RSD %, n=3
0.5- 1000	$Y = 38.82C^{b} + 4221.1$	0.9912	206	0.1	2.15

[a] Preconcentration factor was calculated at 100 µg L^{-1}.
[b] Concentration in µg L^{-1}.

Table 2. Comparison of presented method with other extraction method applied for the extraction and quantification of Celecoxib

Methods	LOD (μg L^{-1})	LDR (μg L^{-1})	Recovery (%)	RSD	Ref.
EADLLME –HPLC/UV[a]	0.1	0.5-1000	94.4	2.15	The current method
LLE-HPLC/UV[b]	3.0	10- 2000	96.0	≤ 10	[6]
LLE-HPLC/UV	6.0	20-2000	85.0	≤ 10.7	[29]
LC-MS/MS[c]	-	5-4000	82.2	≤ 13.0	[30]
SPE-HPLC/UV[d]	7.5	25-2000	91.0	≤ 5.7	[31]
SPE-MIP-HPLC/UV[e]	8.0	50-100000	96.0	≤ 1.1	[5]

[a] Effervescence-Assisted Dispersive Liquid–Liquid Microextraction-high performance liquid chromatography with ultraviolet detection
[b] Liquid- liquid microextraction-high performance liquid chromatography with ultraviolet detection
[c] Liquid chromatography mass mass spectrometry
[d] Solid phase extraction- high performance liquid chromatography with ultraviolet detection
[d] Solid phase extraction- molecular imprinted polymer- high performance liquid chromatography with ultraviolet detection

Comparison of the extraction efficiency of presented method with ultrasonic/vortex/air- dispersive liquid-liquid microextraction methods

In this method, the use of CO_2 bubbles enhanced the dispersibility of organic phase into the sample solution. To confirm this claim, other DLLME techniques such as Air assisted-DLLME (ADDLME), Ultrasound assisted-DLLME (UADLLME), and vortex- assisted DLLME (VADLLME) were examined at optimum conditions. The obtained recoveries (Table 3) showed the high extraction recovery of the presented method with excellent repeatability.

Real sample analysis

The isolation and quantification of CEL in plasma sample was studied to investigate the applicability and accuracy of EADDLE method. The 3 mL of plasma sample were deproteinized by 5 mL of acetonitrile and diluted at the ratio of 1:3 by water and analyzed to assess the EADLLME method at optimal conditions (methanol as an auxiliary solvent; 2 mL, oxalic acid as an acidic promoter; 0.4g, sodium bicarbonate as basic promoter; 0.8g, pH of sample solution 6, chloroform as extraction solvent; 60 μL, and centrifugation time; 5 min). Table 4 lists the recoveries achieved for the plasma spiked at three different concentration levels (10, 50, and 100 μg L^{-1}). The obtained results demonstrated an admissible relationship between the obtained relative recoveries and spiked amount of CEL standards. Fig. 8 shows the data obtained from the proposed method of CEL existing in plasma samples.

Table 3. Comparison of the proposed method with other dispersive liquid-liquid microextraction methods applied for the extraction and quantification of CEL (50 μg L^{-1})

Methods	Recovery (%)	RSD
EADLLME-HPLC/UV[a]	94.4	2.15
UADLLME-HPLC/UV[b]	90.9	3.7
ADLLME-HPLC/UV[c]	96.2	3.1
VDLLME-HPLC/UV[d]	87.5	4.9

[a] Effervescence-Assisted Dispersive Liquid–Liquid Microextraction-high performance liquid chromatography with ultraviolet detection
[b] Ultrasound-assisted liquid-phase microextraction-high performance liquid chromatography with ultraviolet detection
[c] Air-assisted liquid-liquid microextraction- high performance liquid chromatography with ultraviolet detection
[d] Vortex-assisted liquid-liquid microextraction- high performance liquid chromatography with ultraviolet detection

Table 4. Analytical performance of presented method for plasma sample (n=3)

C_{added} (μg L^{-1})	$C_{founded}$ (μg L^{-1})	RR%[a]	RSD[b]	RSD[c]
10	9.8	98	1.87	2.13
50	50.3	100.6	2.27	1.84
100	101.6	101.6	2.05	2.46

[a] Relative recovery
[b] Relative standard deviation, interday (n=3)
[c] Relative standard deviation, intraday (n=3)

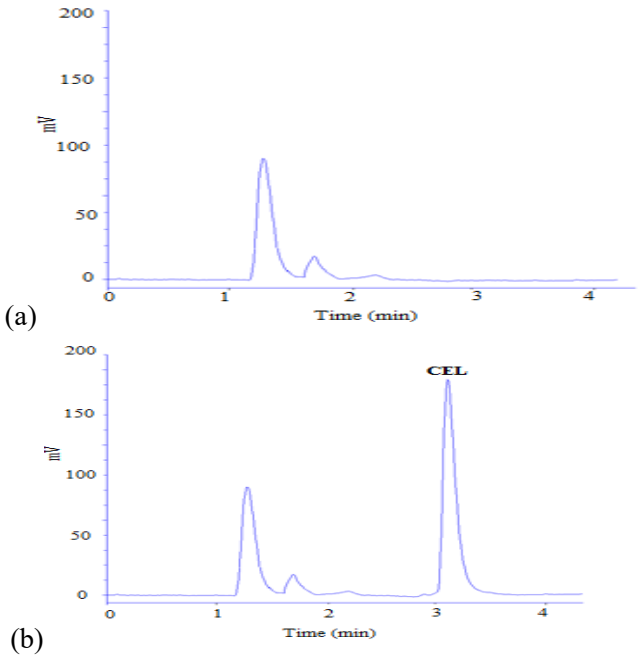


Figure 8. Chromatograms of blank plasma (a) and spiked plasma (b) at 100 μg L^{-1} of CEL

Conclusion

This study addressed the application of effervescence-assisted dispersive liquid–liquid microextraction (EADLLME) through acid-base reaction promotion for the extraction of CEL in the plasma sample and further determination through HPLC/UV. The carbon dioxide produced in the acid-base reaction distributes the extraction solvent throughout the aqueous phase with no complex apparatus. The method offered several advantages over other extraction methods. EADLLME is a simple, fast, accurate, and inexpensive approach with high extraction recovery.

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