Article

Microvolume-DLLME for the Spectrophotometric Determination of Clidinium Bromide in Drug, Urine, and Serum

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The present study combines UV-Vis spectrophotometry and dispersive liquid-liquid microextraction (DLLME) for the preconcentration and determination of trace level clidinium bromide (Clid) in pharmaceutical preparation and real samples. The method is based on ion-pair formation between Clid and bromocresol green in aqueous solution using citrate buffer (pH = 3). The colored product was first extracted using a mixture of 800 µL acetonitrile and 300 µL chloroform solvents. Then, a spectrophotometric measurement of sediment phase was performed at λ = 420 nm. The important parameters affecting the efficiency of DLLME were optimized. Under the optimum conditions, the calibration graphs of standard (Std.), drug, urine and serum were ranged 0.005 - 0.16 µg mL^{-1}. The limits of detection, quantification, and Sandell's sensitivity were calculated. Good recoveries of Clid Std., drug, urine and serum at 0.005, 0.01, 0.1 and 0.16 µg mL^{-1} ranged 93.77 - 101.0%. Enrichment factor was calculated for Std., drug, urine and serum. The method was applied successfully to determine Clid in pharmaceutical preparation and real samples.

Keyword: Clidinium bromide, DLLME, spectrophotometry, microextraction, pharmaceutical preparation.

INTRODUCTION

In the past decade, sample preparation was mainly focused on miniaturization, simplification and automation in order to lower the costs of materials and personnel. Current attempts highly focused on improving the quality of the analytical results. Therefore, cleaning up, concentrating and executing the desired analyte to well-match with the chosen analytical instrument, are the targets of sample preparation techniques [1].

Conventional sample methods, such liquid-liquid extraction [2], Soxhlet extraction [3], distillation and absorption [4,5] are time consuming, difficult to automate and consume large amounts of hazard solvents [6].

The recent trend is toward minimizing the amounts of hazardous solvent consumed, and waste generated, and overcoming the drawbacks of the traditional techniques. Thus, microextraction techniques, such as solid-phase microextraction [7], single drop microextraction [8], liquid-phase microextraction [9], and dispersive liquid–liquid microextraction (DLLME) have immediately attracted a special attention and become widely used in the field of sample preparation [10]. DLLME has been used for the extraction of halogenated organic compounds [11], pesticides [12], palladium [13], and barbituric acid [14].

Spectrophotometric methods are the most common technique. Moreover, simplicity, speed, cost effectiveness, availability of instrumentation, fairly sensitive and precision are what made such methods desirable. Combination of spectrophotometric technique with DLLME-based on ion-pair formation has been applied for the estimation of manganese [15], nitrate [16], fungicide carbendazim [17], and boron [18]. Therefore, in the current study, the determination of clidinium bromide (Clid) in biological samples based on ion-pair has been adopted.

Clid (Figure 1), known as (3-[(hydroxy-diphenylacetyl)-oxy]-1-methyl-1-azoniabicylo-[2.2.2] octane bromide) and widespread as anticholinergic drug can help signs of squirm and abdominal stomach pain by decreasing stomach acid [19]. The methods available for the determination of Clid are high performance liquid chromatography [20,21], capillary electrophoresis [22], and spectrophotometry [23,24].

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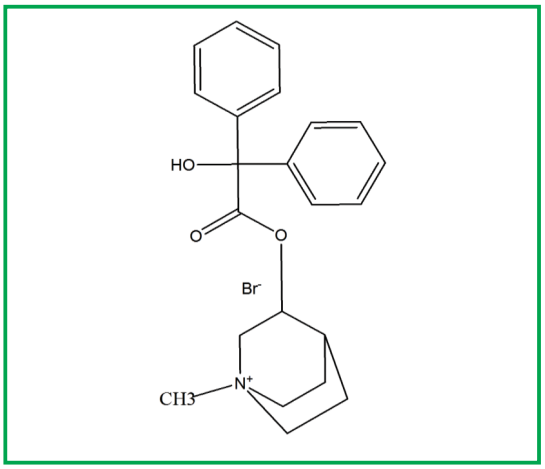

Figure 1. Chemical structure of clidinium bromide.

The aim of the present work is DLLME-spectrophotometric determination of Clid. It is based on the formation of ion pair complex between bromocresol green (BCG) and Clid in an aqueous solution at pH = 3. All parameters affecting the efficiency of DLLME were discussed. The performance of the proposed method was conducted by the determination of Clid in pharmaceutical and real sample.

MATERIALS AND METHODS

Apparatus

APEL PD-303 UV spectrophotometer (Japan) with 1 cm quartz microcells was used. Phase separation process was performed by HERMLE centrifuge (Z -200A) (Germany) using 15 mL centrifuge tubes.

Materials and Solutions

All chemicals and reagents used were of analytical reagent grade. Acetonitrile, chloroform, citric acid, trisodium citrate were supplied from BDH (England) while BCG was purchased from Riedel-de Haën (Germany). Pure clidinium bromide (Clid) was provided by Samarra Drug Industry (Iraq).

A stock solution of 1000 µg mL^{-1} Clid was prepared by dissolving an adequate amount in a least volume of warm distilled water (50 ± 2 ºC). Then, it was cooled and transferred to 100 mL volumetric flask and was diluted to the mark with distilled water.

BCG (10^{-3} M) was prepared by dissolving an appropriate 0.0698 g of the reagent in acetone. Then, it was diluted to 100 mL.

Buffer solution was prepared by mixing 82 mL from citric acid (0.1 M) with 18 mL trisodium citrate (0.1 M) to obtain pH = 3.

Dispersive Liquid-Liquid Microextraction Procedure

An amount of 50 - 1600 µL of Clid standard solution (1 µg mL^{-1}), 1250 µL BCG solution (10^{-3} M), and 5 mL buffer solution were mixed into 15 mL centrifuge tube. Then, the mixture was diluted to 10 mL with distilled water and left for 3 min. A cloudy solution was formed after a rapid injection of a mixture of 800 µL acetonitrile and 300 µL chloroform. The mixture was then centrifuged at 5000 rpm for 5 min. The dispersed fine droplets of the organic phase were collected at the bottom of the tube. After removing the aqueous phase, the organic layer was transferred using a microsyringe. Later, it was placed into the quartz microcell and the absorbance was measured thereafter at 420 nm against the blank solution. The latter was run under the same procedure without adding Clid. All steps of DLLME are summarized in Figure 2.

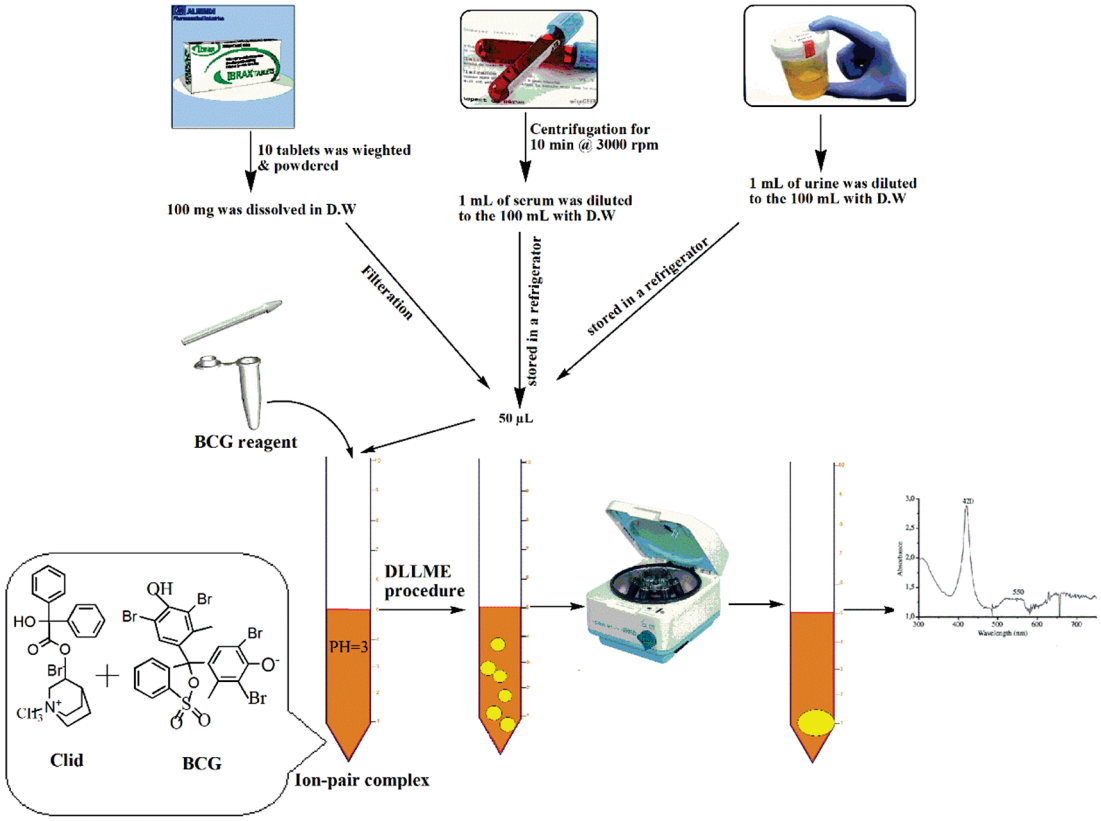

Figure 2. Procedure for the DLLME Method.

Procedure for Real Sample

In the present study, the biological samples were collected from healthy volunteers living in different cities in Baghdad, Iraq. Human serum was separated from the whole blood by centrifuging it at 4000 rpm for 10 min. A 0.5 mL of human serum was spiked with Clid standard (100 µg mL^{-1}), transferred into a volumetric flask, and diluted to 50 mL with distilled water to obtain 1 µg mL^{-1} to be later stored in a refrigerator until use. Human urine was also prepared using same procedure. A 500 µL of serum and/or urine (equivalent to 0.1 µg mL^{-1}) was separately subjected to the DLLME procedure, as described above.

Procedure for Tablets

The contents of 10 tablets of Clid (Labrax tab. contains 2.5 mg) were thoroughly powdered, mixed and the average weight of each one was calculated. An accurately adequate amount of the drug powder was shaken well using 5 mL of warm water (50 ± 2 ºC) for 5 min. The mixture was then transferred into 100 mL volumetric flask, and diluted to the mark using distilled water to get 1000 µg mL^{-1}. A 500 µL of the working solution (equivalent to 0.1 µg mL^{-1}) was separately subjected to the DLLME procedure, as described above.

RESULTS AND DISCUSSION

Effect of Reaction Variables

For the wavelength selection, a sample solution containing different concentration of Clid was tested according to DLLME procedure. The UV-Vis spectra of the sediment phase were recorded in the range of 330 - 600 nm. The maximum Abs spectrum occurs at 420 nm. Thus, 420 nm was selected for all subsequent measurements.

Sample solutions containing different concentrations (0.005 - 0.12 µg mL^{-1}) of Clid were examined according to the recommended procedure for DLLME at 420 nm. Considering Figure 3, the absorbance increased with the increase of Clid concentration.

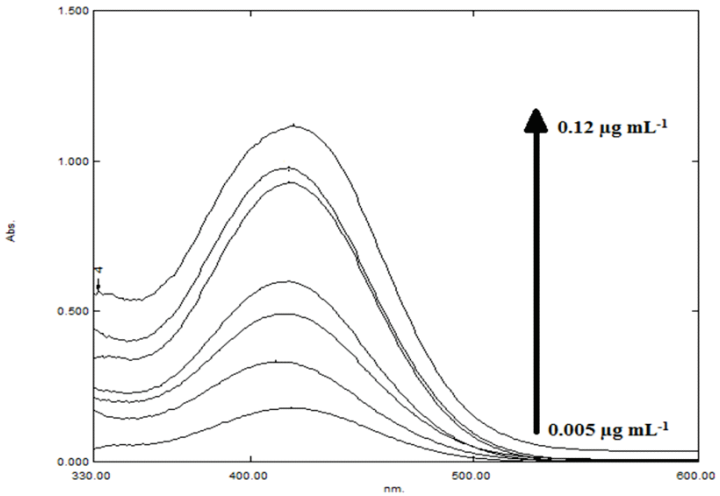

Figure 3. UV-Visible absorbance spectra of sediment phase in the presence of different concentration of Clid.

Clid contains tertiary amine group in its structure (Figure 1). Therefore, in acidic media, an extractable ion pair was formed between positive charged drug and negatively charged reagent (BCG). The theoretical principle of this reaction was based on the dissociation equilibrium of AB electrolyte in aqueous medium according to Eq. 1 (where A^+, the protonated amino drug, and B^-, the BCG reagent, can be shifted to the left (association) if the ion pair is removed by extracting it with water immiscible solvent [24].

$$AB \underset{acidic\ medium}{\longleftrightarrow} A^+ + B^- \qquad (1)$$

The colored product of ion pair is soluble in chloroform. Thus, in this study, spectrophotometric method based on the extraction of this color product using chloroform in the presence of acetonitrile is introduced and optimized in details. Such a factor affects the formation of ion-pair and DLLME method.

The effect of BCG concentration on the intensity of color at the selected wavelengths was investigated with a different concentration of BCG reagent (10^{-5} - 10^{-1} M). Figure 4a shows that the higher absorbance was obtained using 10^{-3} M that was selected as an optimum thereafter.

The reaction between Clid and BCG reagent was carried out in an aqueous buffered solution of pH range between 3 and 6. Higher color intensity was obtained at pH = 3 (Figure 4 b). Therefore, pH = 3 was selected for the further experiment.

The effect of the volume of the selected buffer solution (pH = 3) has also been studied. Figure 4c indicates that 2.5 mL gave high absorbance. Moreover, the time required to complete the formation of ion pair was examined during 1 - 30 min. The results showed that after 3 min, there was no change in color intensity. Therefore, it was chosen for the subsequent experiments.

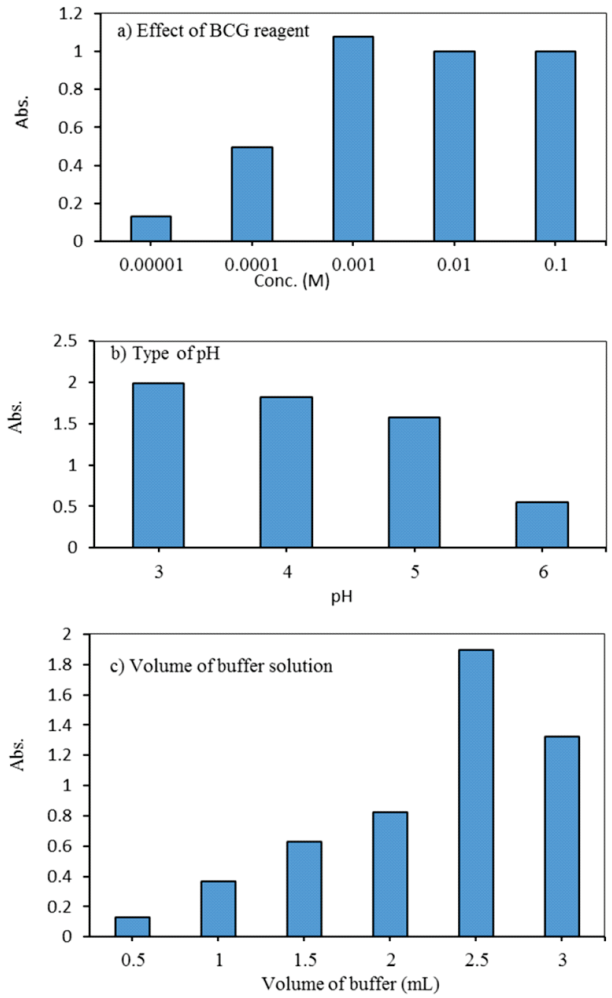

Figure 4. Optimization of Ion Pair: (a) effect of BCG reagent; (b) type of pH; c) volume of buffer solution.

Optimization of DLLME Method

Type of Extraction and Disperser Solvent

With DLLME method, the attention was paid to the selection of an extraction solvent. The solvent should be of low solubility in water, capable to extract the desired analyte, and of low toxicity and volality [11] however, the essential point for the selection of the disperser was its high miscibility in both the extraction and aqueous solutions [6].

For this purpose, several chlorinated solvents (i.e., carbon tetrachloride CCl_4, chloroform $CHCl_3$, methylene chloride CH_2Cl_2 and ethylene chloride $C_2H_4Cl_2$), and low cost and toxic as a disperser solvent (i.e., methanol, acetonitrile, ethanol and acetone) were investigated. Figure 5 shows how the mixture of acetonitrile (800 µL) and chloroform (500 µL) gave higher absorbance. Thus, these solvents were chosen as optimum.

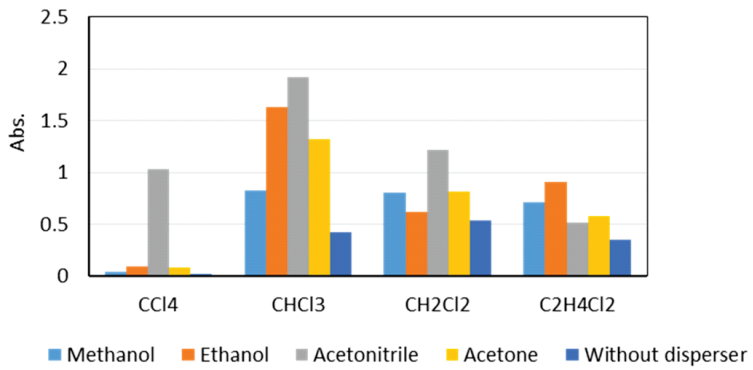

Figure 5. Type of extraction and disperser solvent.

Volume of Extraction and Disperser Solvents

To test the effect of extraction and disperser solvents volume, a series of solutions containing different volumes of chloroform (250 - 500 µL) plus acetonitrile (800 µL) were studied. Figure 6a, shows that when the volume of extraction solvent is increased, the absorbance is increased. Therefore, 300 µL was selected in the next experiment. The effect of the volume of disperser solvent was also examined using various volumes of acetonitrile (600 - 1600 µL), containing 300 µL of the extraction solvent. According to the results obtained in Figure 6b, it was noticed that the absorbance increased upon increase of the volume of disperser solvent from 600 to 800 µL. But it is dropped thereafter probably due to the increase in the solubility of the analyte in the water sample [25]. Thus, 800 µL of acetonitrile was selected.

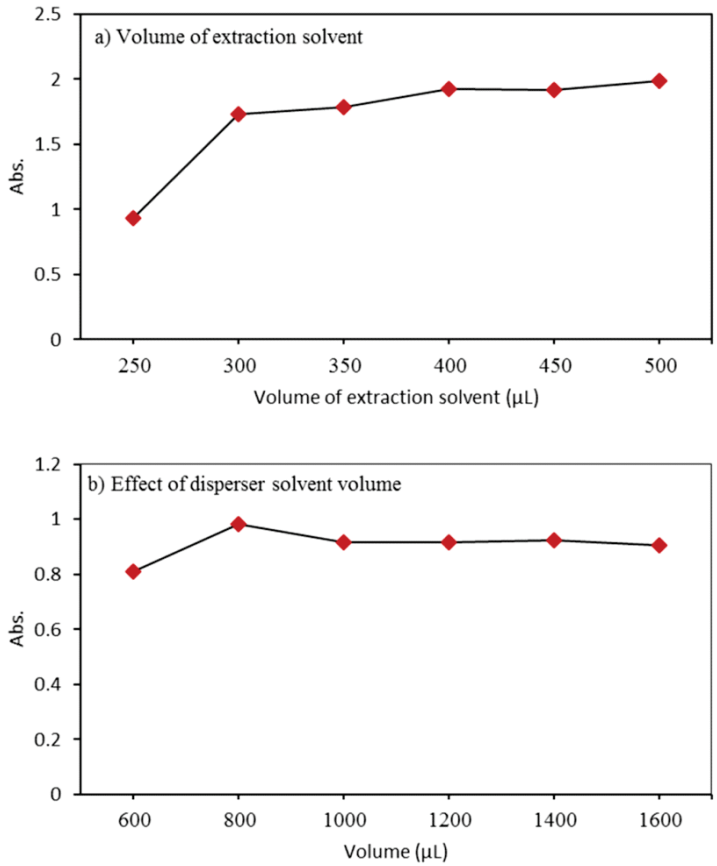

Figure 6. Effect of (a) volume extraction solvent and (b) disperser solvent volume.

Effect of Ionic Strength and Extraction Time

The effect of ionic strength was evaluated by adding different amounts of NaCl (0 - 10%, w/v) into the sample solution. From the results obtained, it was noticed that the absorbance decreased with the increase in the amount of NaCl from 0 to 10% (w/v). This was due to the decrease in the solubility of the extraction solvents in the aqueous solution. Accordingly, the procedure was performed without the addition of salt.

The effect of the extraction time interval time between the injection of the mixture of solvents (extraction and dispersive solvent) in an aqueous sample and the start of centrifugation [16] was studied. The mixture was centrifuged for 1 - 20 min. The results showed that the variations of the absorbance versus the extraction time were not remarkable. This was because the surface area between the extraction solvent and aqueous layer was infinitely large. Thereby, the method was rapid; this was the most important advantage of DLLME technique.

Centrifugation Time and Speed

Centrifugation was required for the separation of the organic solvent from the aqueous phase. To attain the best extraction efficiency, the centrifugation time and speed were optimized in the range of 1 - 10 min and 1000 - 6000 rpm, respectively. The results indicated that the separation was complete within 5 min using a rotation speed of 5000 rpm.

Stoichiometric Relationship

Stoichiometry of the ion pair Clid-BCG complex was established by the molar ratio (Figure 7a) and the continuous variation (Figure 7b) methods. The results showed that the ion-pair had 1:1 (BCG:Clid) ratio.

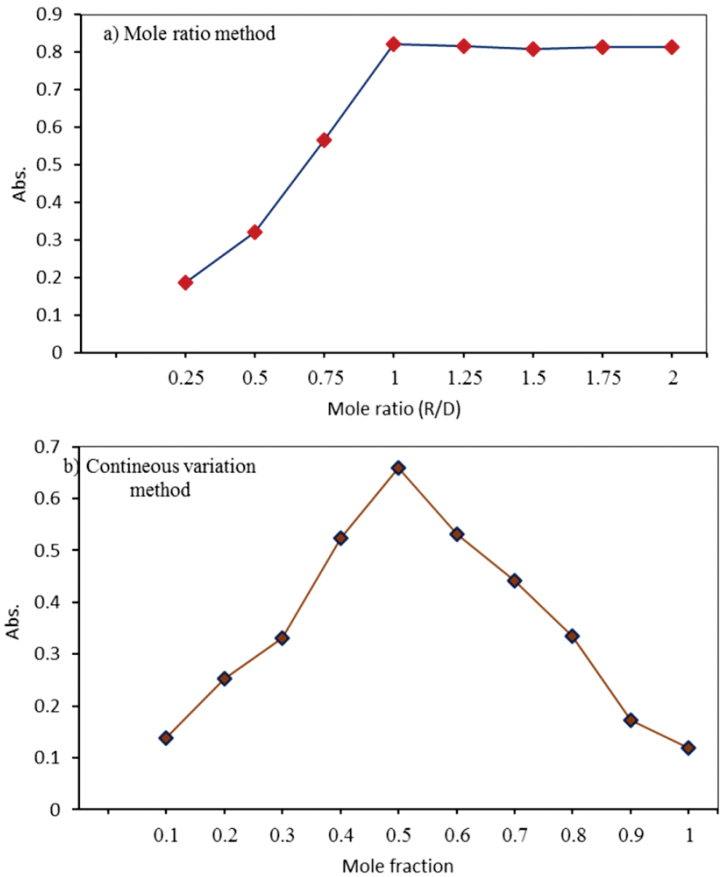

Figure 7. Stoichiometric ratio of Clid-BCG ion pair at λ= 420 nm using (a) molar ratio and (b) continuous variation methods.

Method Validation

Table I summarizes all analytical characteristics of the proposed method. Under the optimum conditions, the linear range was 0.005 - 0.16 μg ml^{-1}. Limit of detection (LOD) and of quantification (LOQ) were calculated using the following equation: $3.S_b/m$ and $10.S_b/m$ respectively, where S_b and m are the standard deviation of the blank and slope of a calibration graph, respectively.

The precision of the method was attributed to its repeatability and reproducibility. Intra-day repeatability and inter-day reproducibility were determined using four different concentrations for Clid Std., drug, urine and serum. Five replicate measurements for each level was conducted. Acceptable precision was reflected from the RSD% values for the intra-day (0.14 - 1.41%) and inter-day (0.16 - 1.66%) (Table II).

The accuracy of the method was performed at four concentration levels for Clid Std., drug, urine and serum. Five replicates were investigated at 0.005, 0.01, 0.1 and 0.16 μg mL^{-1} for each of the samples. The results showed that high recovery values obtained ranged from 93.77 to 101.0% (Table III).

Table I. Analytical performance for the proposed method.

Parameters	Std.		Real samples		
	Before DLLME	After DLLME	Drug	Urine	Serum
Regression equation	0.081x-0.0060	9.9167x+0.1649	6.4659x+0.0953	5.667x+0.0863	8.0413x+0.0833
Correlation coefficient r	0.9992	0.9991	0.9985	0.9984	0.9987
Linearity percentage r^2%	99.84	99.92	99.70	99.68	99.74
Linear range (µg mL^{-1})	10-300	0.005-0.16	0.005-0.16	0.005-0.16	0.005-0.16
ε^a (L mol^{-1} cm^{-1})	$3.49x10^4$	$4.29x10^6$	$2.8x10^6$	$2.45x10^6$	$3.48x10^6$
LOD^b (µg mL^{-1})	0.75	0.0042	0.0042	0.0048	0.0041
LOQ^c (µg mL^{-1})	2.95	0.0141	0.0139	0.0159	0.0137
S^d (µg cm^{-2})	$1.2x10^{-2}$	$1x10^{-5}$	$1.5x10^{-4}$	$1.8x10^{-4}$	$1.2x10^{-4}$
^{E}Enrichment factor	……..	122.0	80	70	100

[a] Molar Absorptivity; [b] Limit of Detection; [c] Limit of Quantification; [d] Sandells Sensitivity; [E] Enrichment Factor was calculated from the slope of calibration curve after and before DLLME.

Table II. Intra-day repeatability and Inter-day reproducibility of the proposed method.

Conc. (µg mL^{-1})	Intra-day repeatability ([a]RSD%, n=5)				Inter-day reproducibility ([b]RSD%, n=15)			
	Std.	Drug	Urine	Serum	Std.	Drug	Urine	Serum
0.005	1.13	1.41	0.89	0.99	1.23	1.66	1.16	1.27
0.01	1.24	1.09	1.13	1.30	1.38	1.24	1.29	1.60
0.1	0.23	0.44	0.53	0.28	0.26	0.54	0.66	0.30
0.16	0.14	0.27	0.32	0.22	0.16	0.36	0.41	0.26

[a]RSD five determinations; [b]RSD Average of five determinations over three days.

Table III. Accuracy of the proposed method

Samples	Conc. (μg mL^{-1})		Recovery% (E%)
	Added	Found	
Std.	0.005	0.0048	95.40 (-4.61)
	0.01	0.0096	95.90 (-4.10)
	0.1	0.097	97.40 (-2.60)
	0.16	0.162	101.01 (1.01)
Drug	0.005	0.0048	94.96 (-5.04)
	0.01	0.0097	96.66 (-3.34)
	0.1	0.099	98.60 (-1.41)
	0.16	0.159	99.28 (-0.72)
Urine	0.005	0.0047	94.23 (-5.77)
	0.01	0.0095	94.76 (-5.24)
	0.1	0.099	99.22 (-0.78)
	0.16	0.159	99.40 (-0.60)
Serum	0.005	0.0047	93.77 (-6.23)
	0.01	0.0095	94.64 (-5.36)
	0.1	0.097	96.86 (-3.14)
	0.16	0.159	99.98 (-0.02)

Effect of Interference

The influence of potential compounds and ions was established using of 0.01 μg mL^{-1} of Clid in drug, urine and serum samples. The results are illustrated in Table IV. Under the reaction conditions, all the interference ions and compounds did not interfere.

Table IV. Effect of foreign ion on Clid determination.

Coexisting ions	Tolerance ratio
Starch, Glucose, Sucrose, Lactose, Creatinine, urea, uric acid, SO_4^{-2}, K^+, Ca^{+2}, PO_4^{-3}	1:1000
Starch, Glucose, Sucrose, Lactose, Creatinine, urea, uric acid, SO_4^{-2}, K^+, Ca^{+2}, PO_4^{-3}	1:100
Starch, Glucose, Sucrose, Lactose, Alanine, Creatinine, urea, uric acid, SO_4^{-2}, K^+, Ca^{+2}, PO_4^{-3}	1:10

Application

The proposed method was applied for the extraction and determination of Clid in three different pharmaceutical preparations and biological samples: spiked with Clid and pharmaceutical preparations. As shown in Table V, the theoretical content of the active ingredient represents the actual amount of Clid in the pharmaceutical tablet. On the other hand, the data obtained in the practical content reflects the amount of Clid in the selected tablets after applying the proposed method.

Table V. Determination of Clid in three pharmaceutical preparation using the proposed method.

Sample Nº	Pharmaceutical tablet content and manufacturer (2.5 mg)	Weight of pharmaceutical equivalent to 0.02 µg of active ingredient (g)	Theoretical content of active ingredient (mg)	Practical content of active ingredient (mg)	E%	Efficiency of determination % ± RSD%
1	Librax, Swaziland	0.3020± 2.252	2.5	2.53	1.155	101.2±0.23
2	Libraxam, Iraq	0.1794± 3.005	2.5	2.44	-2.610	97.39±0.36
3	Eipco, Egypt	0.3096± 9.526	2.5	2.51	0.211	100.2±0.30

The spectra of Clid for pure, drug, urine and serum samples before and after using DLLME are illustrated in Figure 8. The result indicates that there was a significant enrichment of the analyte after undergoing the DLLME method.

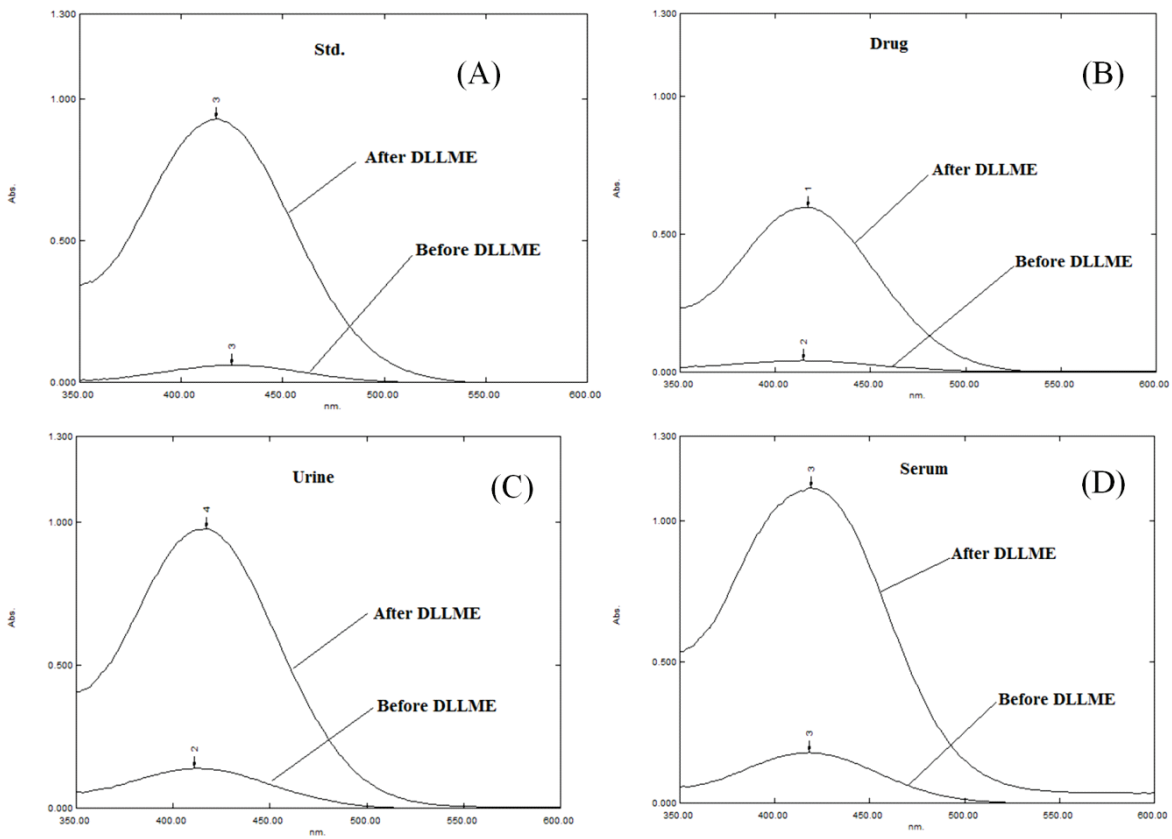

Figure 8. Spectrum of Clid spiked with 0.1 µg mL^{-1} before and after DLLME for (A) Std.; (B) Drug; (C) Urine; and (D) Serum.

The proposed method was compared with other reported methods regarding the determination of Clid (Table VI). The result showed the advantages of the proposed method with respect to the linear ranges, LOD and LOQ.

Table VI. Comparison of linear range, LOD and LOQ for the determination of Clid using proposed and other methods.

Method	Linearity range ($\mu g\ mL^{-1}$)	LOD ($\mu g\ mL^{-1}$)	LOQ ($\mu g\ mL^{-1}$)	Samples	Ref.
HPLC	2.5-300	0.088	0.294	Pharmaceutical preparation	19
Capillary electrophoresis	5-100	1	4	Drug	22
LLE/UV-Vis	10-300	0.75	2.95	Pharmaceutical preparation	24
DLLME/ Spectrophotometry	0.005-0.16	0.0042	0.0141	Std.	Present study
		0.0042	0.0139	Drug	
		0.0041	0.0137	Serum	
		0.0048	0.0159	Urine	

CONCLUSION

Compared to the LLE method, the proposed method was revealed to be simple, sensitive, rapid, reproducible, and requires a low solvent volume. In addition, it helped reduce the hazardous chlorinated solvent consumption and provide a high enrichment factor. Moreover, the method outlined a successful application and permitted the separation and preconcentraion of Clid at a trace level in biological and pharmaceutical samples.

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