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Development of a Dispersive Liquid-Liquid Microextraction for the Spectrophotometric Determination of Penicillin G in Milk and Biological Samples

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ABSTRACT

Background: This study developed a dispersive liquid-liquid microextraction technique followed by spectrophotometric analysis for preconcentrating/determining penicillin G benzathine and penicillin G potassium.
Methods: The procedure was based on forming a color complex of penicillin G with bromothymol blue and its extraction by DLLME. The univariate method was employed for optimizing the parameters.
Results: Optimized conditions for extracting benzathine and potassium penicillin G were 1.5 mL methanol and acetone as dispersive solvents; 300 µL $CHCl_3$ and 250 µL CCl_4 as extracting solvents; pH 4 and 3; 0.8 mL and 0.9 mL bromothymol blue (10-3 M), and NaCl 0.8 M for both analytes. The calibration curves were linear over the ranges 50-750 and 800-950 IU mL^{-1} for analyzing benzathine, and 800-1600 and 1750-6500 IU mL^{-1} for potassium penicillin G. The relative standard deviations and detection limits were obtained to be 6.53% and 0.101 IU mL^{-1} and 4.65% and 0.149 IU mL^{-1} for determining benzathine and potassium penicillin G, respectively.
Conclusion: The proposed method was employed for extracting and determining penicillin in human plasma, urine, orange juice, and milk, with acceptable recovery values of 94.3-102%.

1. Introduction

Many antibiotics are produced and used to treat bacterial diseases in humans and animals [1]. The excessive use of antibiotics has led to bacterial resistance and created a serious problem in clinical treatment [2]. In addition, residual antibiotics enter groundwater, surface water, wastewater, and sludge, resulting in environmental pollution and increasing bacterial resistance [3]. Wastewater produced in pharmaceutical industries and their discharge to the effluent cause also the accumulation of these pollutants in the environment and water sources [4]. These threats confirm the importance of monitoring and measuring antibiotics in environmental samples. Penicillin G was the first discovered antibiotic by humans and used in the clinical treatment of bacterial diseases affected by streptococci, staphylococci, and spirochetes. This antibiotic prevents the synthesis of bacterial cell walls by binding to penicillin-binding proteins of bacteria [5]. Penicillin G is fabricated as a water-soluble (i.e., its potassium salt) and water-insoluble (i.e., as its benzathine compound) species (Figure 1). Determining Penicillin G was investigated by employing an extraction method such as solid phase extraction (SPE) or liquid-liquid extraction (LLE) coupled with spectroscopic [6, 7] or electrochemistry techniques [8, 9]. However, the widespread use of this antibiotic in humans and livestock

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motivate us to investigate the more sensitive and selective methods for determining this compound in foods and biological samples. Therefore, a variety of sample preparation/preconcentration techniques have been assessed. Dispersive liquid-liquid microextraction (DLLME) is one of these methods, which was first introduced by Rezaei et al. (2006) [10, 11]. Several factors have contributed to the popularity of this method, including its simplicity, speed, low cost, environmental friendliness, minimal use of organic solvents, high enrichment factor, and selectivity. Further, the related research results in its remarkable development [12, 13]. In this method, an extraction solvent, preferably with higher density than water and immiscible with water as the extractant, such as carbon tetrachloride, chloroform, and dichloromethane, and a dispersing solvent that is miscible in both the extraction phase and the aqueous phase like methanol, ethanol, acetonitrile, and acetone is employed. The solvents are mixed and rapidly injected into an aqueous sample solution, creating a turmoil that causes the formation of cloudy emulsion droplets. The aqueous samples cause the fine-formed droplets to disperse and the analyte is extracted into the droplets of the extraction solvent. The cloudy mixture was centrifuged and the fine droplets were sedimented down at the bottom of the test tube and removed by a syringe for analysis [14, 15]. We chose this method for analyzing different analytes such as drugs [16, 17], pesticides [18], herbicides [19], organic samples [20, 21], and heavy metals [22, 23] in complex matrices because of its compatibility with powerful analytical instruments such as gas chromatography [24, 25], liquid chromatography (LC) [26, 27], ion mobility spectrometry [28], and atomic absorption spectrometry [29]. The present study aims to investigate the preconcentration and determination of penicillin G benzathine and penicillin G potassium in human plasma, urine, orange juice, and milk. The antibiotics measurements were followed spectrophotometrically with bromothymol blue reagent. The affecting parameters on the processes were optimized using "one at a time" methods. The figures of merit were assessed for all of the real samples.

2. Materials and Methods

Penicillin G benzathine (1.2 million I.U.) and penicillin G potassium (5 million IU) were prepared from Jaber Ebne Hayyan Pharmaceutical Company. The stock solutions of the antibiotics were prepared by dissolving the antibiotics in deionized water. Chloroform (≥ 99%, Merck), dichloromethane (≥ 99%, Merck), dichloroethane (≥ 99%, Merck), carbon tetrachloride (≥ 99.5%, Merck), as extractant solvents and methanol (99.6%, Dr. Mojallali), ethanol (100%, Merck), acetonitrile (100%, Merck) and acetone (99%, Dr. Mojallali) were used as disperser solvents. Bromothymol blue, sodium chloride (≥ 99%), sodium hydroxide (extra pure), acetic acid (≥ 99.8%), formic acid (98-100%), and phosphoric acid (85%) were obtained from Merck Chemical Company. All the buffer and working solutions were prepared in deionized water. The solutions were stored at 4°C in the dark. Calcium chloride (≥ 97%, Fluka) and ammonium sulfate (≥ 99%, Merck) were utilized in the real sample pretreatment.

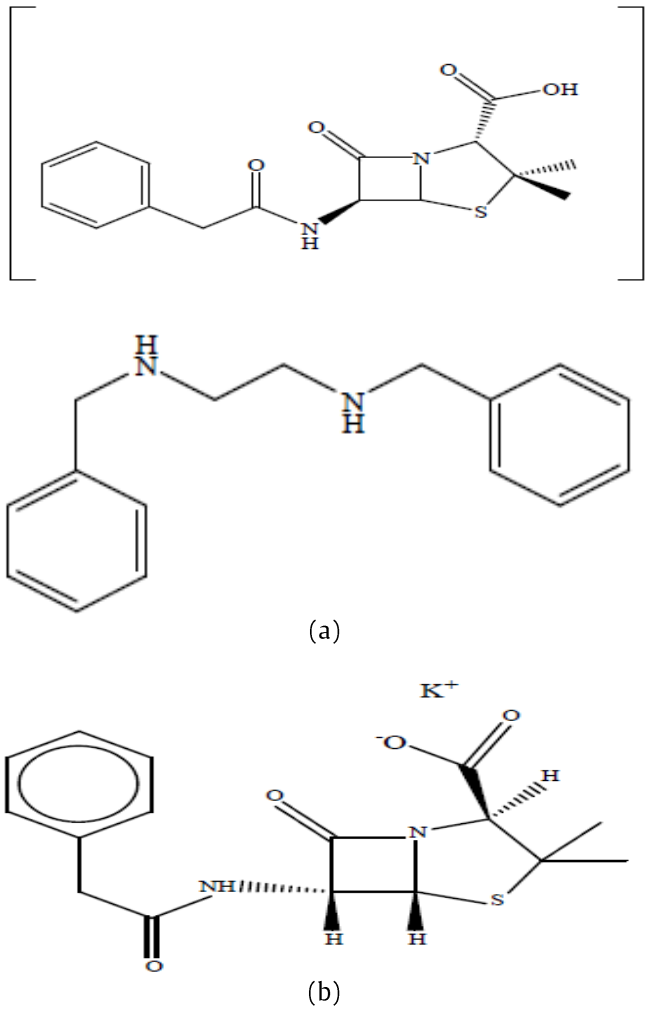

Figure 1: Chemical structure of penicillin G benzathine (a) and penicillin G potassium (b).

2.1 Instrumentation

Analysis of penicillin G benzathine and penicillin G potassium was carried out using bromothymol blue as reagent at 412 nm and 330 nm, respectively, by a Shimadzu UV-Vis spectrophotometer (model 160) and a rectangular cell with a path length of 1.0 cm. A centrifuge, Heraeus Labofuge 300, was used to separate phases. A Metrohm pH meter (model 780) was employed for pH measurements. A syringe and an insulin syringe were utilized for adding solvent and sampling from the test tube.

2.2 Dispersive liquid-liquid microextraction procedure

5 mL of a penicillin G (benzathine (800 IU mL^{-1}) or potassium (3330 IU mL^{-1})) solution, 1 mL acetate buffer solution with specific pH and 0.8 or 0.9 mL, 10^{-3} M

bromothymol blue were placed into an extraction vessel. An aliquot of 1.5 mL of a disperser solvent (methanol or acetone) containing extraction solvent (300 µL chloroform for penicillin G benzathine and 250 µL carbon tetrachloride in penicillin G potassium extraction, respectively) was rapidly injected into the solution using a 2 mL syringe. The cloudy solution produced was centrifuged for 5 min at 3000 rpm. The centrifugation results in the sedimentation of the extraction solvent in the bottom conical test tube [30]. The volume of the sedimented phase was about 150-200 µL. The 5 µL of sedimented phase obtained from penicillin G benzathine was removed using a micro syringe, diluted with chloroform and analyzed spectrophotometrically in 412 nm. The sedimented phase was isolated from penicillin G potassium diluted by acetone and analyzed at 330 nm [31]. All tests were repeated three times. Figure 2 displays a graphical depiction of the experimental method. The enrichment factor (EF) was defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration of analyte (C_0) within the sample [32]. Penicillin G concentration in the sedimented phase was obtained by plotting the calibration curve of the standard solution in a UV-vis spectrophotometer [32].

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

The extraction recovery (R %) was calculated by using Eq. (2):

$$\mathrm{R}\ (\%) = \frac{n_{sed}}{n_0} \times 100 = \frac{C_{sed} \times V_{sed}}{C_0 \times V_{aq}} \times 100 = \left(\frac{V_{sed}}{V_{aq}}\right) \times EF \times 100 \quad (2)$$

where n_0 and n_{sed} are the total analyte and amount of analyte extracted to the sedimented phase, respectively. V_{sed} and V_{aq} are the volumes of the settled phase and the sample solution, respectively.

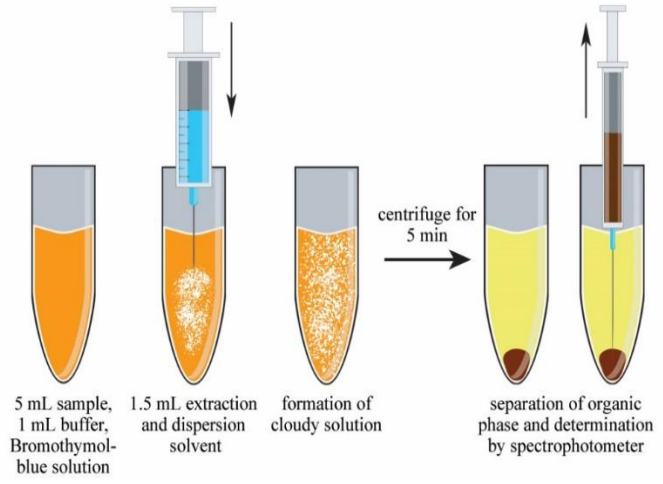

Figure 2: Schematic representation of the experimental procedure

2.3 Real sample preparation

The proposed DLLME method was applied to determine penicillin G in human plasma, urine, orange juice, and milk. Zanjan Blood Transfusion Organization prepared the plasma sample. Saturated ammonium sulfate was added to the sample to precipitate protein, centrifuged at 4000 rpm for 40 min, and filtered. The urine sample was collected from a volunteer person. Industrial orange juice was purchased from the local supermarket. The raw milk was prepared from the dairy industry. Calcium chloride (1 M) was added to the milk sample and then heated for 30 min. After that, it was filtered. All the samples were centrifuged for 40 min (4000 rpm) and passed through a membrane filter. The penicillin G was spiked to the obtained solutions and tested by the same DLLME methods.

3. Results and Discussion

There are various parameters affecting dispersive liquid-liquid extraction processes. The type and volume of the extraction and disperser solvents, pH of aqueous solution, ligand concentration, and ionic strength are the most critical parameters that must be optimized for efficient extraction.

3.1 Selection of extraction solvents

Choosing a suitable organic extraction solvent is a major factor for a DLLME process. Some properties, such as the capability for extracting target compounds, low solubility, and higher density than water, should be considered in this process. Chloroform, carbon tetrachloride, dichloroethane, and dichloromethane were chosen and investigated as extraction solvents. To choose the suitable extraction solvent, the recovery of penicillin G benzathine or penicillin G potassium from 5 mL samples buffered by 1 mL solution (pH 5 for penicillin G benzathine and pH 2 for penicillin G) containing 1 mL bromothymol blue and 1.5 mL of acetone as disperser solvent was investigated using 250 µL of each examined extraction solvent. The results are shown in Figure 3. According to the results, the extraction performed by using chloroform and carbon tetrachloride presented the highest recovery ability; therefore, they were selected as an extraction solvent for the extraction of penicillin G benzathine and penicillin G potassium extraction, respectively. The observed different efficiency of the process by varying types may be due to the difference in the polarity of the analytes. The more hydrophilic nature of penicillin G benzathine causes its dissolution in chloroform.

3.2 Selection of disperser solvent

The polar solvents, including acetone, acetonitrile, ethanol, and methanol which are miscible in aqueous solution and extraction solvent, were used to choose appreciate disperser solvents [13]. The recovery of penicillin G from the investigated samples solution was tested by 1.5 mL of the mentioned disperser solvents (Figure 4). The best result was obtained by methanol and acetonitrile as disperser solvents for penicillin G benzathine and penicillin G potassium extraction. However, due to the broadening of the absorption peak when using acetonitrile as an extraction solvent, acetone was selected to continue the study.

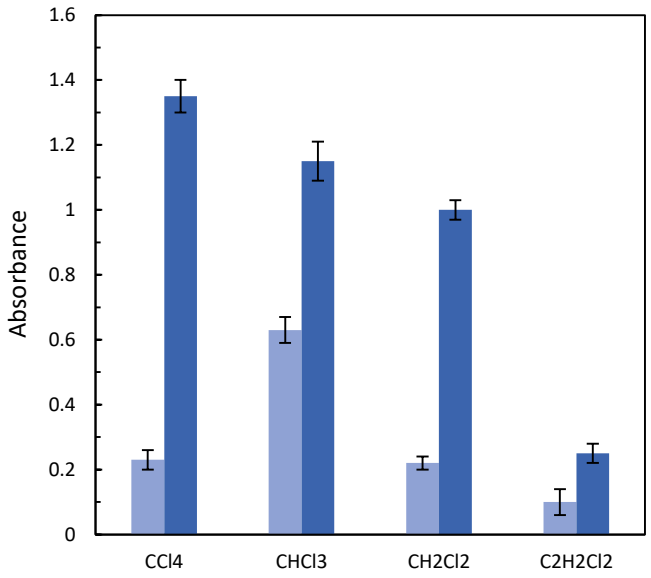

Figure 3: Efficiency of different extraction solvents (250 µL) evaluated for extraction of 5 mL penicillin G benzathine 800 IU mL^{-1}, 1.5 mL disperser solvent (acetone), 1 mL buffer pH 5, 1mL bromothymol blue 10^{-3} M (black column) and 5 mL penicillin G potassium 3330 IU mL^{-1}, 1.5 mL disperser solvent (acetone), 1 mL buffer pH 2, 1mL bromothymol blue 10^{-3} M (gray column).

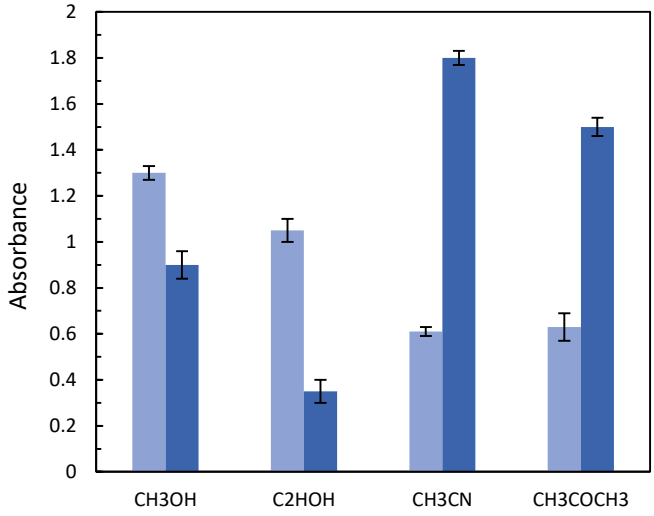

Figure 4: Efficiency of different dispersion solvents (1.5 mL) evaluated for extraction of 5 mL penicillin G benzathine 800 IU mL^{-1}, 250 µL solvent extraction ($CHCl_3$), 1 mL buffer pH 5, 1mL bromothymol blue 10^{-3} M (black column) and 5 mL penicillin G potassium 3330 IU mL^{-1}, 250 µL solvent extraction (CCl_4), 1 mL buffer pH 2, 1mL bromothymol blue 10^{-3} M (gray column)

3.3 Extraction solvent volume

In order to optimize the volume of the extraction solvent, the recovery of penicillin G benzathine and penicillin G potassium by using different volumes of chloroform and carbon tetrachloride as extraction solvents (100-400 µL) with the total volume of the disperse and extraction solvents 1.5 mL and same DLLME procedure were examined (Figure 5). The results showed that the absorption increases by increasing the volume of carbon tetrachloride from 100 to 300 µL and variation chloroform in the range of 100 to 250 µL. The observed decrease in absorbance beyond the given values is attributed to the increase in the sediment phase and the decrease in the enrichment factor. Also, by using higher volumes of the extraction solvent and a decrease in the volume of dispersed solvent, the cloudy solution did not form well because the smaller volume of dispersion solvent cannot disperse the particles well. Therefore, the resulting particles become larger, and the contact surface becomes less, so the extraction efficiency decreases.

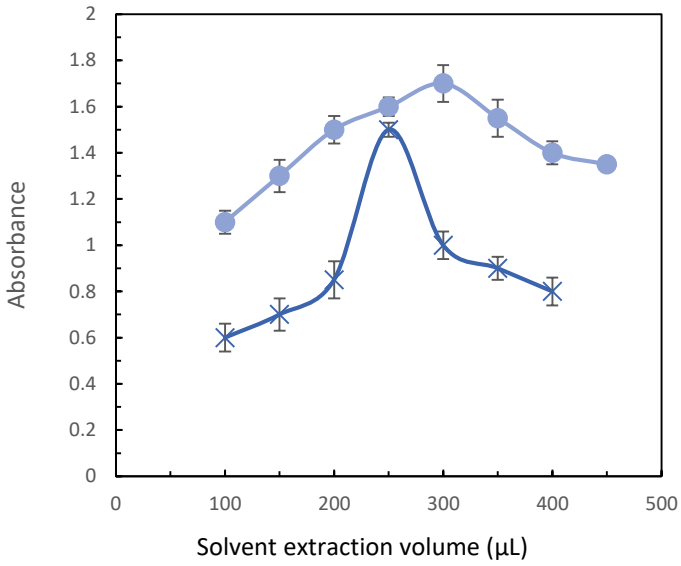

Figure 5: Effect of solvent extraction (chloroform for penicillin G benzathine and carbon tetrachloride for penicillin G potassium) on the recovery of penicillin G benzathine (800 IU mL^{-1}) (•) and penicillin G potassium (3330 IU mL^{-1}) (✖). The experimental conditions are given in the caption of Figure 4.

3.4 Effect of pH on the Process

Sample solution pH is an important factor affecting the efficiency of the process. The sample solution pH controls the ionic or neutral form of penicillin G (pK_a= 2.75), which can be extracted from an organic solvent [33]. Extraction experiments were performed to evaluate the optimum pH of the sample solutions by adjusting pH values in the 2-6 by adding 1 mL of formate, acetate, or phosphate buffer to sample solutions. Figure 6 shows that the penicillin G benzathine and penicillin G potassium recovery increases by pH up to 4 and 3, respectively. The recovery decreases beyond this value.

3.5 Bromothymol blue volume effect

Figure 7 illustrates the results of the volume of the bromothymol blue (10^{-3} M) as the extractant on the extraction efficiency of two types of penicillin G. As expected, the absorption and extraction efficiency is increased with ligand volumes to 0.8 mL for penicillin G benzathine and 0.9

mL for penicillin G potassium. A further increase in the volume to 1.6 mL decreased the extraction efficiency due to the extraction of the ligand into the extracting solvent and its saturation. Thus, the volume of 0.8 and 0.9 mL of 10^{-3} M bromothymol blue solution was chosen as the optimal ligand volume for liquid-liquid extraction of penicillin G dispersion.

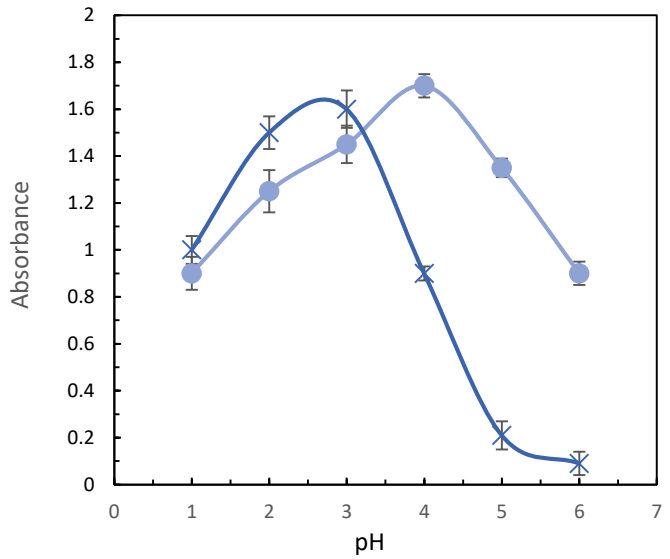

Figure 6: Effect of pH on the absorbance of 5 mL penicillin G benzathine 800 IU mL^{-1}, 1.5 mL methanol containing 300 µL $CHCl_3$, 1 mL buffer with different pH, 1mL bromothymol blue 10^{-3} M (•) and 5 mL penicillin G potassium 3330 IU mL^{-1}, 1.5 mL acetone containing 250 µL CCl_4, 1 mL buffer with different pH, 1mL bromothymol blue 10^{-3} M (✖)

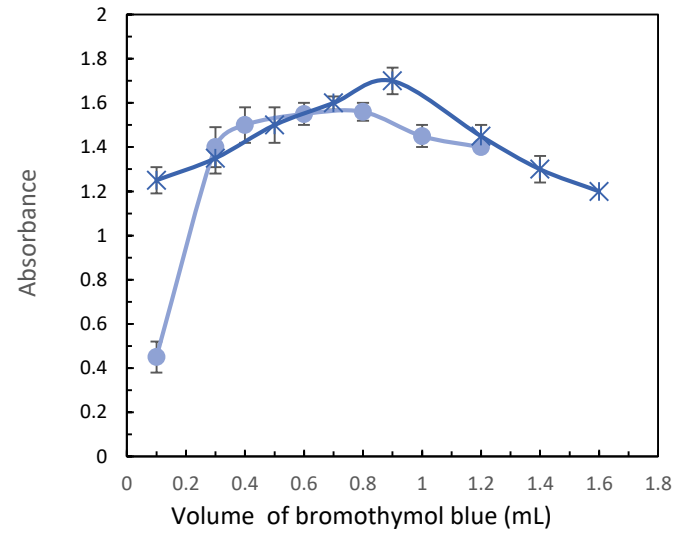

Figure 7: Effect of the bromothymol blue (10^{-3} M) volume on the absorbance of penicillin G benzathine 800 IU mL^{-1} (•) and penicillin G potassium 3330 IU mL^{-1} (✖). The experimental conditions are given in the caption of Figure 6.

3.6 Effect of ionic strength on the Process

The effect of ionic strength on the studied extraction process was assessed using aqueous solutions containing NaCl in the 0.4-1 M range while other test conditions were kept constant. Figure 8 presents the recovery of the studied analytes enhanced with electrolyte concentration up to 0.8 M, and beyond this value, the extraction efficiency was diminished. The increase in absorption is probably due to the effect of salting out, which results in the augmentation of the extraction of the hydrophobic complex into the organic phase. Further, the increase in the salt concentration increases the sedimented phase volume and consequently reduces absorption. Hence further studies were performed in the 0.8 M of NaCl.

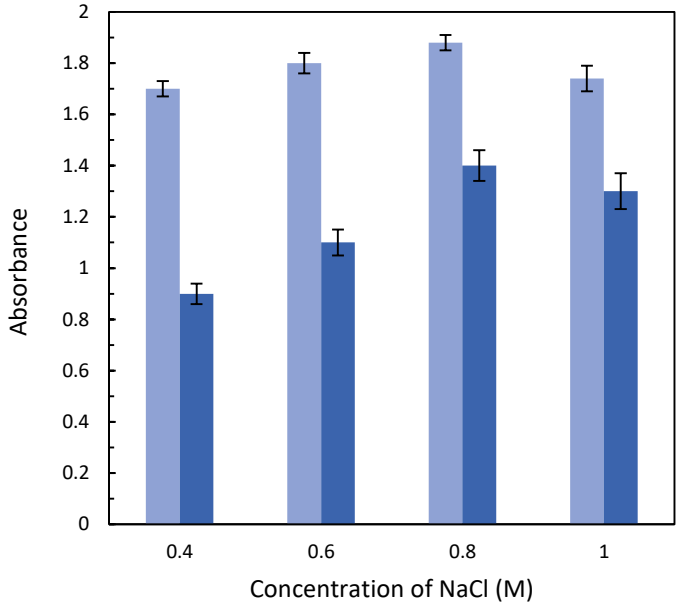

Figure 8: Effect of salt addition on the recovery of penicillin G benzathine (black column) and penicillin G potassium (gray column). The experimental conditions are given in the caption of Figure 6.

3.7 Analytical performance

To evaluate the usefulness of the extraction processes for quantitative analysis of the target analytes in aqueous samples, we studied the analytical performance of the proposed methods and validated in terms of linearity, the limit of detection (LOD), the limit of quantification (LOQ), and relative standard deviation (RSD%). Calibration curves were obtained by carrying out a series of extraction experiments in the optimum conditions. The calibration curve of the proposed method in the extraction of penicillin G benzathine was linear in two ranges. The first calibration curve was in the concentration range of 50-750 IU mL^{-1} (A = 0.0023 C-0.0212, R^2 0.9984, where A and C are absorbance and concentration of penicillin G benzathine in IU mL^{-1}, respectively) and the second linear range was between 800-950 IU mL^{-1} (A = 0.0012 C + 0.76, R^2 0.9936,). The corresponding limit of detection 0.101 IU mL^{-1} was obtained based on three times the standard deviation of the blank signal to the slope of calibration curve ratio ($3s_{bl}/m$) by performing eight replicate determinations.

Table 1: Analytical characteristics of the proposed method for extraction of Penicillin G benzathine and Penicillin G potassium.

Analyte	Equation	R^2 [a]	L. R[b] (IU mL^{-1})	RSD% (n=5)	LOD (IU mL^{-1})
Penicillin G benzathine	A = 0.0023C-0.0212	0.9984	50-750	6.53	0.101
	A = 0.0012C+0.76	0.9936	800-950	6.14	
Penicillin G potassium	A = 0.0007 C+0.5154	0.9965	800-1600	4.65	0.149
	A = 0.0002 C+0.8776	0.9902	1750-6500	1.47	

[a] Correlation coefficient. [b] Linearity range.

Table 2: Results of the recovery of penicillin G benzathine and penicillin G potassium. in real samples[a].

Analyte[a]	Sample	Equation	R^2 [a]	L. R (IU mL^{-1})	Added (IU mL^{-1})	Recovery (%)
Penicillin G benzathine	milk	A = 0.0021C-0.2837	0.9961	250-600	300	98.8±1.5
	orange juice	A = 0.0027C+0.1848	0.9929	100-600	300	101.1±0.9
	urine	A = 0.0003C+0.6609	0.9587	100-600	300	94.3±1.7
	plasma[b]	-	-	-	-	-
Penicillin G potassium	milk	A = 0.0005 C-0.1834	0.9962	800-1600	1300	99.2±2.0
	orange juice	A = 0.0005 C+0.0812	0.9982	800-1600	1300	102.0±0.8
	plasma	A = 0.0002C-0.037	0.9928	100-600	300	97.5±1.9
	U rine[c]					

[a] Experimental conditions: 5 mL sample solution containing penicillin G benzathine, 1.5 mL methanol containing 300 µL $CHCl_3$, 1 mL buffer pH 4, NaCl 0.8 M, 0.8 mL bromothymol blue 10^{-3} M and/or 5 mL sample solution containing penicillin G potassium, 1.5 mL acetone containing 250 µL CCl_4, 1 mL buffer pH 3, NaCl 0.8 M, 0.9 mL bromothymol blue 10^{-3} M. [b] In the plasma samples in the range of 800-1600 IU mL^{-1} of penicillin G benzathine calibration curve wasn't linear. [c] In the urine samples in the range of 100-600 IU mL^{-1} of penicillin G potassium calibration curve wasn't linear

Table 3: Comparison of analytical characteristics of the presented method with some other reported methods

Preconcentration method	Analysis method	Recovery (%)	RSD (%)	LOD (µg L^{-1})	Ref
molecularly imprinted polymer (MIP)	LC-UV	95-96	3.8-8	0.75 (milli Q water) 1.7 (tap water)	[34]
SPE	HPLC-UV	91.0 -123%.	N.R.	0.49	[35]
Solid phase microextraction	HPLC-UV	81-100.7	N.R.	0.06-.0.26	[36]
SPE-membrane	Raman	N.R.	N.R.	0.9	[37]
Molecularly imprinted solid-phase extraction	HPTLC	95-108	N.R.	5	[38]
ion-paired extraction	HPLC-UV	> 82	≤ 2	1-2	[39]
DLLME	UV	97.5-101.1	4.65[a] 6.53[b]	0.093[a] (0.15 IU ml^{-1}) 0.076[b] (0.10 IU ml^{-1})	This work

[a] data for penicillin G potassium, [b] data for penicillin G benzathine

In order to evaluate the repeatability of the method, a series of experiments were carried out five times during the same day at a concentration level of 300 and 800 IU mL^{-1} and the relative standard deviations (RSD %) were calculated to be 6.53% and 6.15%, respectively. In the case of the extraction of penicillin G potassium, the calibration curve was linear in two ranges of concentrations. The first line was in the concentration range 800-1600 IU mL^{-1} (A = 0.0007C + 0.5154, R^2 0.9965) and the second linear range was between 1750-6500 IU mL^{-1} (A = 0.0002C + 0.8776, R^2 0.9902). The corresponding LOD was obtained at 0.149 IU mL^{-1}. The relative standard deviation at a concentration level of 1000 IU mL^{-1} (n = 5) was 4.65% and at the concentration level of 3330 IU mL^{-1} was 1.47% (Table 1).

3.8 Applicability of the proposed method

In order to assess the applicability of the DLLME method in the extraction of penicillin G from real samples, the proposed method was applied for the extraction of injected penicillin G benzathine (100-600 IU mL^{-1}) and penicillin G potassium (800-1600 IU mL^{-1}) from milk, an orange juice, a urine sample, and a plasma samples. Table 2 presents the results of three replicated extraction experiments under optimum conditions. Recovery rates ranged from 94.3-102%, indicating acceptable accuracy for the proposed method. These results confirm that the sample matrices had little effect on the DLLME procedure.

3.9 Comparison of the proposed methods with some other reported procedures

Table 3 compares some figures of merits of the proposed methods based on the DLLME with some previously published methods for preconcentrating and determining penicillin [34-39]. The results revealed that the percentage recovery by the studied methods is comparable to or higher than that reported by the compared methods. Molecularly imprinted polymer (MIP) [34], SPE [35], SPE with membrane [37], molecularly imprinted solid-phase extraction [38], and ion-paired extraction [39] are the only methods that showed a higher limit of detection for penicillin in the current study, except for solid-phase microextraction [36]. Furthermore, this study used spectrophotometry to detect penicillin, a very simple, low-cost, and rapid method compared to HPLC-UV or Raman spectroscopy.

4. Conclusion

DLLME was developed for preconcentrating penicillin G benzathine and penicillin G potassium before spectrophotometric determination using bromothymol blue reagent. We chose chloroform in methanol and carbon tetrachloride in acetone as the extraction and dispersion solvent for benzathine and potassium penicillin G, respectively. The method was simple, rapid, cost-effective, sensitive, reproducible, and environmentally friendly. Figures of merit of the presented method were evaluated and showed that the calibration curve in two concentration ranges was linear for both analytes. The detection limit according to $3s_{bl}/m$ was obtained at 0.101 and 0.149 IU mL^{-1} for benzathine and potassium penicillin G, respectively. The proposed method efficiently extracted the analytes from human plasma, urine, orange juice, and milk samples.

Authors' Contributions

Leila Dolatyari: Data analysis; Writing original draft. Parinaz Gharavi Khiavi: Field investigation; Laboratory investigation. Mohammad Reza Yaftian: Conceptualization; Study design; Supervision.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

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