RESEARCH ARTICLE

Analysis of Amoxicillin in Pharmaceutical Preparations and Biological Samples Based on Micro-cloud Point Technique Prior to Spectrophotometric Determination

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ABSTRACT

A simple, green, and cost-effective method for the trace determination of amoxicillin (AMX) based on micro-cloud point extraction (MCPE) prior to spectrophotometric determination has been demonstrated. To extract AMX using MCPE method, the procedure was carried out using Triton X-114 as a non-ionic surfactant. The effects of main parameters influencing the extraction efficiency such as surfactant and salt concentrations, solution pH, temperature and incubation time were inspected and optimized. Under optimum conditions, good analytical features were obtained. A calibration curve was found to be linear in the concentration range of 0.02 – 3.50 µg mL^{-1} with low detection limit (0.05 µg mL^{-1}), good precision with the relative standard deviation of < 2.3%, and high recoveries in the samples (> 96.5%). The obtained results determine the proposed methodology is applicable in satisfactory manner to determine the AMX in pharmaceutical preparations and biological samples.

Keyword: Amoxicillin, Cloud point method, Micro, Pharmaceutical preparations.

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INTRODUCTION

Amoxicillin (AMX) is an antibiotic known as (2S,5R,6R)-6-[[(2R)-2-amino-2-(4-hydroxyphenyl) acetyl] amino]-3,3-dimethyl-7-oxo-4-thia-1azabicyclo [3.2.0] heptane-2- carboxylic acid with an empirical formula of C16H19N3O5S(Figure 1).[1] AMX was started being used for medical purpose in 1972, 14 years after being discovered in 1958.[2,3] Kept on the WHO's list of essential, AMX is used as the treatment of infections, including acute otitis media, streptococcal pharyngitis, pneumonia, various skin infections, urinary tract infections (UTIs), Salmonella infections, early-stage Lyme disease, and chlamydia infections.[1-4]

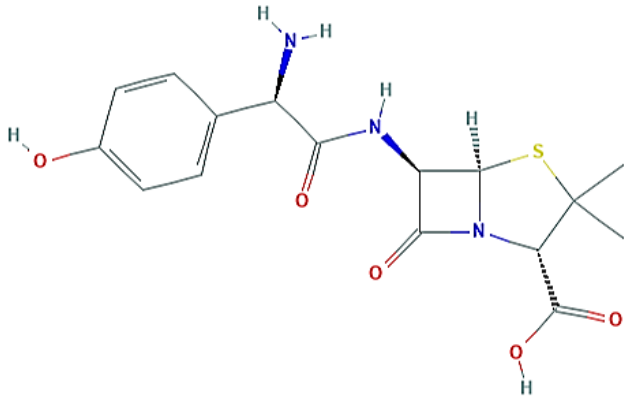

Figure 1: Chemical structure of AMX

The side effect of AMX is similar to those for other β-lactam antibiotics, including nausea, vomiting, possible loose bowel movements,[5] rashes, and antibiotic-associated colitis. In addition to that, some rare but adverse effects include mental alteration conditions, lightheadedness, insomnia, distraction or lack of concentrations, anxiolytic behaviour, susceptibility towards lights and sounds, and ambiguous thinking.[6] Even by treating with combination of amoxicillin/clavulanic acid for more than one week has induced mild hepatitis in some patients. Acute overdoses of AMX has been ingested in young children with lethargy, vomiting, and in some cases even renal dysfunction.[5,6]

With the recent developments, analytical chemistry's current scenario is moving towards more user-friendly and miniaturized instruments specifically designed for better quality control. More user-friendly and cost-effective techniques have enabled segmented usage of sample preparation methods for selective extraction of targets. These methods with preconcentration and clean-up operations enable

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the analysis of several classes of matrices with more particular and better detection boundaries.

Separation methods based on cloud point extraction (CPE) are the practical application of surfactants in analytical chemistry and have become an alternative to solvent extraction. Compared with conventional solvent extraction, CPE which is widely used for separation, purification and preconcentration of various substances, uses surfactants and avoids using a huge quantity of toxic, expensive, and flammable organic solvents.

Various methods have been published for the determination of AMX in pharmaceutical formulations and biological fluids such as HPLC,[7-12] colorimetric,[13] capillary electrophoresis,[14] and spectrophotometry.[15-17]

This study is aimed to develop a smooth and responsive CPE method for determining AMX in various samples by using spectrophotometry detection. The method is used for extraction, cleaning-up and preconcentration of AMX from aqueous samples with the help of Triton X-100, identified as extracting solvent. The influences of main parameters on the extraction efficiency of AMX were investigated and optimized. At last, a comparison of figures of merit of the proposed was done with several reported methods in the literature.

Experimental

Apparatus

A Shimadzu 1800 UV-Vis spectrophotometer (Germany) with 0.5-cm quartz microcells was used in the wavelength range of 190–1100 nm.

Phase separation process acceleration was performed using a HERMLE centrifuge (Z-200A) (Germany) with 10 mL centrifuge tubes. Thermostatic water bath shaker, Thermolyne digital, JEIO TECH (Korea) also has been used.

MATERIALS AND SOLUTIONS

All chemicals and reagents used were of analytical reagent grade. Anhydrous sodium carbonate, sodium nitrite, glucose, lactose, starch, *ethylene di amine tetra acetic acid,* and sucrose were supplied from BDH (England). Hydrochloric acid and *p*-amino benzoic acid were purchased from Fluka (Germany). Alanine, ethanol, urea and creatinine were supplied by Sigma (USA).

A 1000 μg mL^{-1} stock solution of AMX (Samarra Drug Industry, Samarra, Iraq) was prepared by dissolving 0.10 g of Amoxicillin trihydrate using 5 mL of ethanol. The solution filtered to remove any undissolved residue and the volume was complete to 100 mL using deionize water. The solution was stable for at least 2 weeks at 4ºC and the series of working standard solution (50–500 μg mL^{-1}) was freshly prepared by dilution from stock standard solution.

2×10^{-3} M of p-amino benzoic acid (PABA) was prepared by dissolving 0.0275 g in a little volume of deionized water. The solution filtered to remove any undissolved residue and the volume was completed to the mark (100 mL) with deionize water.

2×10^{-3} M of sodium nitrite was prepared by dissolving 0.0138 g in deionize water transferred into 100 mL (volumetric flask) and completed to the mark with deionized water.

M of hydrochloric acid was prepared by diluting (13 mL) of 11.64 M of concentrated hydrochloric acid with deionized water in 100 mL volumetric flask.

2 M of anhydrous sodium carbonate was prepared by dissolving 21.2 g in 10 mL deionized water transferred into 100 mL volumetric flask and completed to the mark with deionize water and mixed well.

10% (v/v) of Triton X 114 solution was prepared by diluting with 10 mL in hot deionized water and completed to the 100 mL in volumetric flask.

0.1%, w/v of Ethylene diamine tetraacetic acid was prepared by dissolving 0.1g in deionized water and completed to 100 mL in a volumetric flask.

Procedure for the Cloud Point Extraction Determination of AMX

Into 10 mL of a volumetric flask that placed in an ice bath (0-5°C), 1 mL of PABA solution (2×10^{-3} M), 1 mL of sodium nitrate (2×10^{-3} M) was added and mixed gradually. Then, 1 mL of HCl (1.5 M) solution was added immediately (to justify the acidity and remove the residual nitrite (nitric acid)).

After that, 0.3 mL of AMX (500 μg mL^{-1}) was added, and complete to the mark with deionize water. The solution was later transferred to 15 mL centrifuge tube. 1.5 mL of 2 M anhydrous sodium carbonate solution was added and the solution was diluted to 10 mL with deionize water.

After that, the solution was stood for 15 minutes at room temperature and 1.0 mL of (10%) v/v of Triton X114 and 1.0 mL of 0.10% w/v of EDTA the colored azodye. The latter was heated for 35 minutes at 45ºC and centrifuged for 5 minutes at 4000 rpm after cooling for 5 minutes in an ice bath. The cloud point was obtained and separated at the bottom (i.e., the surfactant-rich phase becomes viscous)—yellow azodye. After elimination of the aqueous phase, the organic layer (500 μL) was removed using a microsyringe then, placed into 0.5 cm quartz microcell and absorbance was measured against blank solution (that treated but without the addition of AMX) at $\lambda = 435$ nm. Figure 3.2 summarizes the steps of the suggested CPE procedure.

Procedure for the Determination of AMX in Vial and Capsule

To 100 mL volumetric flask, two vials of AMX (each vial contain approximately 0.5 g AMX). A 0.05 g of the drug powder was dissolved using deionized water, filtered through a Whatman No. 1 filter paper, 100 mL volumetric flask, then diluted to the mark with deionized water subjected to analysis by recommended CPE procedure (mentioned above).

Into 100 mL volumetric flask, 10 capsules of AMX (each capsule containing approximately 0.511 g AMX) were weighed, powdered, and mixed. A 0.05 g of the drug powder was dissolved in deionized water, filtered through a Whatman No. 1 filter paper, then diluted to the mark with deionized water and subjected to analysis by the recommended CPE procedure.

Procedure for the Preparation of Biological Samples

Biological samples were collected from five healthy individual's volunteers living in Baghdad, Iraq. For the preparation of human serum, 1mL of serum samples was centrifuged at 3000 rpm for 5 minutes. After that, the serum was collected and diluted to the mark (100 mL) with deionizing water. While preparing human urine, 1 mL of urine sample spiked with AMX 5 ppm; then transferred into 100 mL volumetric flask and diluted to the mark with deionized water. A 5 µL aliquots of the spiked urine samples were separately subjected to the CPE procedure as described above.

RESULTS AND DISCUSSION

Absorption Spectra

The diazotization reaction between para-aminobenzoic acid with sodium nitrite was performed in acidic media to form diazonium salts. The later was coupled with amino group of AMX in alkaline media to form the azodye[15] Scheme 1 presents the coupling reaction mechanism. The colored azo dye insoluble in deionized water.

Therefore, in the current study, a spectrophotometric method is based on the diazonium reaction of this colored azo dye product using the micro cloud point extraction based on Triton X-114 as a surfactant. The absorbance spectra of the complex after CPE has shown that the maximum absorbance spectra at $\lambda = 435$ nm. Therefore, all the absorbance measurements were performed at 435 nm (Figure 2).

Optimization of reaction conditions and MCPE conditions for the determination of AMX

To obtain the maximal extraction efficiency (high recovery), important experimental parameters that can potentially influence the enrichment performance, such as pH of sample solution, salt concentration, concentration of the surfactant, and centrifugation speed have been investigated in detail for MCPE methods. A series of experiments were designed for this goal, as discussed below. For every experiment, the analysis was replicated at least 3 times.

Effect of acid

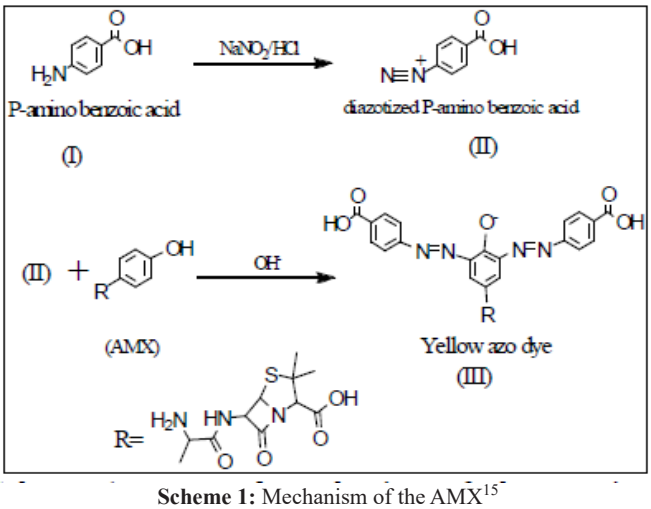
Most commonly, diazonium salts are prepared by treatment of aromatic amines with nitrous acid and additional acid. Usually the nitrous acid is generated *in situ* (in the same flask) from sodium nitrite and the excess mineral acid (usually aqueous HCl, H_2SO_4, or any mineral acids).[19]

To study the effect of acid on the diazonium reaction, 0.5 mL of different types of acid (0.5 M) such as HCl, H_2SO_4, and H_3PO_4 were examined. The results show that hydrochloric acid gave the best absorbance that was chosen for all subsequent experiments.

Effect Concentration and Volume of HCl

A series of different concentrations of 0.5 mL HCl (0.5, 1, 1.5, 2, and 2.5 M) were studied. Figure 3 shows that the absorbance increases gradually with increasing concentration until reaching 1.5 M; then its decrease thereafter.

Differnt volumes of 1.5 M of HCl (i.e., 0.5, 1.5, 2, and 3 mL) were studied. It was found that 1.0 mL gave the highest absorbance. So, 1 mL of 1.5 M was selected as an optimum for further experiments.



Scheme 1: Mechanism of the AMX[15]

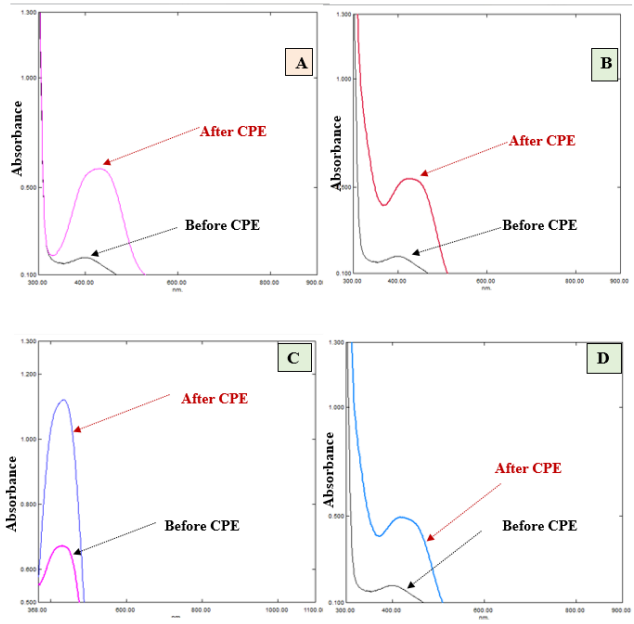


Figure 2: Absorption spectra of (15.0 µg mL^{-1}) A: Stander AMX before CPE and Absorption spectra of (15.0 µg mL^{-1}) of AMX developed method (after CPE) B: Drug C: Serum D: Urine

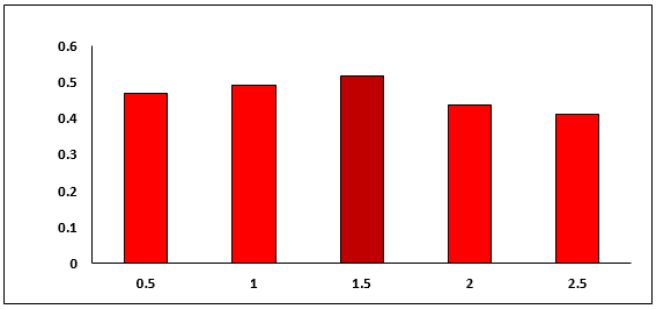


Figure 3: Effect of concentration of HCl on the diazotization reaction

Effect of type of bases

The effect of the different type of bases on coupling reaction color intensity were conducted using 1 M of sodium hydroxide, ammonium hydroxide, potassium hydroxide, and sodium carbonate. The results (Figure 4) show that sodium carbonate gave the highest absorbance that was chosen for further experiment.

Effect of Sodium Carbonate Concentration

The effect of different concentrations of sodium carbonate on the coupling reaction (to formed diazonium salts) was studied using 1-mL of a series of 0.5, 1, 1.5, 2, 2.5, 3, and 4 M. According to the results obtained, the absorbance increases gradually till 2 M, then it decreases thereafter.

The effect of different volumes of sodium carbonate was performed using different volumes of sodium carbonate ranged 0.5-3 mL It was found that 1.5 mL gave highest absorbance. Therefore, 1.5 mL of 2 M was chosen for further experiments as optimum.

Effect of Concentration of Triton X-114 and its Volume

A successful CPE maximizes the extraction efficiency by minimizing the phase volume ratio, and the surfactant concentration is the main parameter affecting the phase ratio.[18] Thus, the effect of different concentrations of Triton (TX-114) on the formation of azo dye formation were investigated using range of 1 to 20.0 % (v/v). It was found that the absorbance increases with increasing the concentration of Triton X-114 up to 10.0% (v/v) (Figure 5). While using a concentration of more than 10.0% of surfactant (TX-114), the sensitivity decreases due to dilution of the sample by additional surfactant solution.

The effect of different volumes of 10% (v/v) Triton X-114 was studied at the range of (0.5–2 mL). The results shown that the absorbance increase by increasing the volume of Triton X-114 up to 1.0 mL. Thus, 1.0 mL of 10% (v/v) of Triton X-114 was chosen for the rest of our experimental work.

Effect of Addition of Chelating Agent EDTA

The selection of chelating agent is the regulating factor for all metal-CPE schemes.[19,20] Several chelating agents have been used to produce sufficiently hydrophobic complexes to be isolated in the surfactant-rich phase of a micellar solution. The effect of addition of different concentration of EDTA from 0.02–0.2 (% w/v) were studied. The absorbance of the complex was significantly increase with increasing the concentration of EDTA from 0.02 to 0.10 % (w/v), and gradually decreasing at the concentration more than 0.10% (W/V).

To study the effect of EDTA (0.10 w/v concentration), volume was performed using different volume ranged from 0.5–2 mL on the absorbance. The azodyze absorbance significantly increased with increasjng the volume of EDTA from 0.5 to 1 mL, and gradually decreased at the volume more than 1 mL. Therefore, 1 mL of 0.10 % (w/v) EDTA was chosen as the optimum volume for further experiments.

Effect of Equilibration Temperature and Time

Generally, in analytical analysis, employing experimental conditions at lowest temperature and shortest reaction time is preferred.[21]

The incubation time and equilibrium temperature above the cloud point should be calculated correctly. Effects of temperature and reaction time were investigated at the range of 2 to 55°C and 10 to 50 minutes, respectively. All the results shown in Figure 3.14, 3.15 revealed that maximum reaction rate was obtained at 45°C within 35 minutes. Above 55°C the separated organic phase disappears.

Effect of Centrifuge Speed and Time

Incubation time and matching temperature are two essential parameters in CPE. According to the optimum experiments to preconcentrate trace levels of AMX- diazotized *p*-amino benzoic acid with high extraction yield in short time. The centrifugation time and speed were evaluated in the range of 1–60 minutes and 1500–5000 rpm, respectively and further cooling in different times of 1–10 minutes has been realized. So, the best centrifugation time and speed were 5 min at 4000 rpm, respectively and cooling for 5 min in ice bath leads to high recoveries of AMX - diazotized *p*-amino benzoic acid in short time. Thus, all the subsequent experiments were performed at 4000 rpm for 5 minutes, and cooling for 5 minutes in ice bath.

Salting-out Effect

In this study, the effect of external salting has been studied by adding different concentrations of several salts such as, NaCl, Na_2SO_4, KCl and $NaNO_3$ in the concentration ranged from 1-20% (w/v). It was observed that there is a decrease in signal

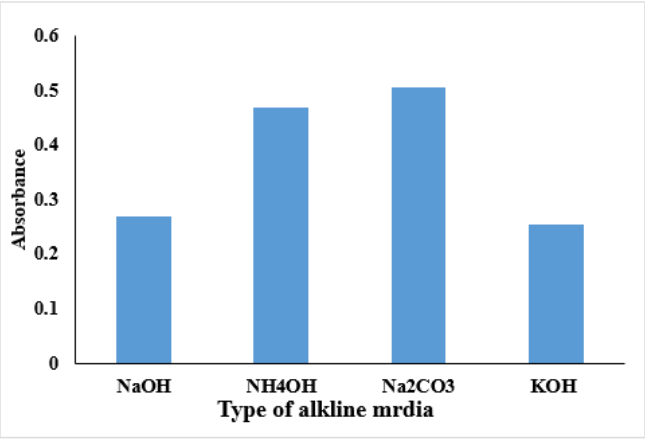


Figure 4: Effect type of the alkaline media on the coupling reaction

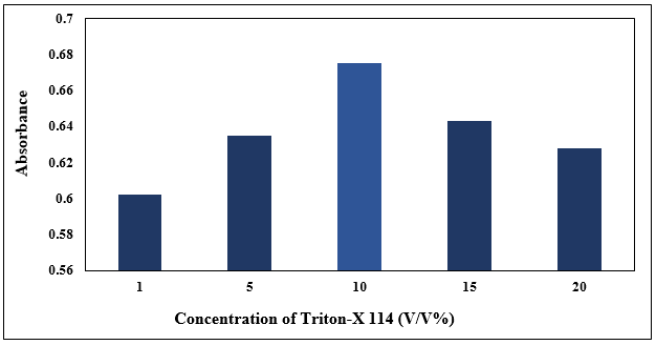


Figure 5: Effect concentration of Triton-X 114 (%V/V)

Table 1: Analytical parameters for CPE method

	Standard		*Real samples*		
Parameters	*Before CPE*	*After CPE*	*Drug*	*Serum*	*Urine*
Regression equation	0.0218x + 0.0862	0.595x + 0.1198	0.5732x + 0.1294	0.5567x +0.1435	0.5784x + 0.1527
Correlation coefficient, r	0.9999	0.9999	0.9997	0.9997	0.9999
Linear range (μg mL^{-1})	0.5–80.0	0.02–3.50	0.02–3.50	0.02–3.50	0.02–3.50
Ɛ [a] (L mol^{-1} cm^{-1})	9.26×10^3	2.59×10^5	2.52×10^5	2.7×10^{-5}	2.64×10^5
LOD[b] (μg mL^{-1})	0.30	0.01	0.05	0.05	0.015
LOQ[c] (μg mL^{-1})	1.01	0.03	0.017	0.01	0.05
S[d] (μg cm^{-2})	1.15×10^{-5}	1.0×10^{-5}	7.1×10^{-5}	-2.0×10^{-5}	1.9×10^{-5}
PF [f]	10	24	24	25	24

[a] Molar absorptivity, [b] Limit of detection, [c] Limit of quantification, [d] sandell's sensitivity, [f] Preconcentration factor= the ratio of volume of sample (mL) and volume of organic layer (mL).

Table 2: Accuracy and precision of the proposed method

Concentration	*Intra-day repeatability (RSD [a] %, n=5)*				*Inter-day reproducibility (RSD%, n=15)*				*Recovery [b] % (n=5)*			
(ng mL^{-1})	*Standard*	*Drug*	*Serum*	*Urine*	*Standard*	*Drug*	*Serum*	*Urine*	*Standard*	*Drug*	*Serum*	*Urine*
20.8	1.15	1.21	0.56	1.45	2.0	1.75	1.22	1.5	98.6	98.9	96.7	98.4
104	0.78	1.7	1.61	1.3	2.12	2.3	1.25	0.94	98.9	99.6	97.6	99.5
1041	0.3	0.24	0.44	0.7	0.37	0.54	0.43	0.35	99.9	99.1	100.4	98.9
2500	0.21	0.11	0.19	0.18	1.08	0.93	0.21	0.2	99.5	99.7	98.9	99.3

[a] Relative standard deviation, [b] Average of five determinations

intensity as salt concentration increases. This is attributed to viscosity increase for bulk solution that results in a decrease in diffusion rate that tends to restrict the analyte movement from the aqueous solution to the organic phase. Hence, further optimization was performed without salting-out.

Interference Studies

The effect of interference compounds on the determination of AMX was investigated. Sample solutions containing 21 ng mL^{-1} of AMX and different concentrations of other possibly existing compounds were prepared according to the developed procedure. The results show that most of the selected compounds (lactose, sucrose, starch, alanine and glucose, urea) did not interfere even if found at 10, 100 or 1000-fold excess over the analyte.

METHOD VALIDATION

Calibration Curve for the CPE Method

Calibration graph of the standard drug, serum and urine were prepared at the optimum conditions by plotting absorbance versus concentration of AMX. Analytical features of the proposed method, including regression equation, linearity, correlation coefficient r, linearity percentage r2 %, and detection limit (LoD), limit quantification (LoQ) are summarized in Table 1. LoD and LoQ were calculated based on 3Sb/m and 10Sb/m respectively, (when Sb and m are stander deviation of the blank and slope of the calibration graph, respectively)

Accuracy and Precision

The repeatability and reproducibility of the proposed method was evaluated by processing five replicates of five different concentrations on the same day (intra-day) and over three days (inter-day). The results shown in Table 3 indicated that the proposed method was accurate and precise depending on recovery %Rec and relative standard deviation %RSD.

Recoveries were adopted for serum and urine samples spiked with five different AMX standard concentrations and subjected to the proposed CPE method. Good recoveries for real samples in the range of 98.4–99.9 were obtained (Table 2).

Application

The performance of the developed method was evaluated by applying the proposed CPE for the determination of AMX level in different samples (different pharmaceutical preparations, serum and urine). For the determination of AMX contents in pharmaceutical preparations, the procedure adopted on weight of 0.05 g from each drug in order to obtain 0.025 μg mL^{-1} (this concentration was chosen as centroid of the calibration curve). Analytical results (%Rec.) for determination of AMX in different capsules formulations obtained from two origins are illustrated in Table 3.8. Obtained results by proposed CPE method shown applicability of this method for the determination of trace levels of AMX suggesting the capability of developed CPE method to overcome matrix effects. For the analysis of serum and urine samples, the results (Table 3) show the proposed CPE method's applicability to recover AMX in urine samples.

Evaluation of the Proposed Method

The results obtained were statistically compared with those obtained from British pharmacopeia's, using the student t-test and variance ratio F-test at 95% confidence level.[22] It was found

Table 3: Determination of AMX in pharmaceutical preparation using CPE

Sample No.	*Pharmaceutical capsule content and manufacture (500 mg)*	*Weight of pharmaceutical equivalent to 0.05 µg of active ingredient (g) ±RSD%*	*Practical content of active ingredient (mg)*	*Efficiency of determination % ± RSD%*
1	Giomax UAE	0.511 ± 0.06	511	98.9 ± 0.95
2	Cipmox (India)	0.532 ± 0.05	532	99.48 ± 1.12
3	AMX	0.507 ± 0.07	507	101.1 ± 1.3

Table 4: Comparison of proposed method with standared method using T- and F- test.

Drug from 500mg	*Proposed method*	*Standard method*	
	Rec.% $(Xi)_1$	*Rec.% $(Xi)_2$*	*$(Xi_1 - Xi_2)$*
AMX – Pure	98.6	98.0	0.6
Giomax UAE	98.3	98.1	0.2
AMX(SDI)	101.1	98.4	2.7
Cipmox (India)	98.8	97.2	1.6
S_1^2 =1.3 S_2^2=0.2			
$(Xi_1 - Xi_2)^-$	1.3		
Sd	1.1		
T expt	2.3		
F expt	6.27		

T-theoretical at 95% confidence limit = 2.78, F-theoretical = 6.39

$(Xi_1 - Xi_2)$: Average, Sd: standard deviation of $(Xi_1 - Xi_2)$

not to differ significantly between the proposed method and the official method. In all cases, the calculated t and F-values, shown in Table 4, indicate that there is no important difference between either method in accuracy and precision in the AMX determination in pharmaceutical preparations.

CONCLUSION

A fast, economical, effective, and easy to operate method based on Micro-cloud point extraction method for the preconcentration and determination of traces of AMX is presented. Triton X-114 was used as a non-ionic and green extractant solvent. Compared to the similar extraction methods, MCPE showed comparable LoDs for the selected compound, while it is much faster. The reported methods for the determination of AMX mostly depend on using HPLC, which is an expensive instrument using pure toxic organic solvent as mobile phase.

Moreover, a coupling of the suggested MCPE method with spectrophotometry is equipped with microcells, as an inexpensive, fast and available instrument; therefore, a successful minimizing of toxic organic solvents consumption and increasing sensitivity for the determination of the target analytes was obtained. Since spectrophotometric instrumentations are simple, inexpensive, and mostly available in common laboratories, the proposed MCPE method is applicable in ordinary laboratories without need of expert personnel.

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