

Simultaneous Estimation of Amoxicillin Trihydrate, Cloxacillin Sodium and Probenecid in Tablet Formulation by UV-Spectrophotometric Method

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Abstract Amoxicillin trihydrate, cloxacillin trihydrate and probenecid are proven for their therapeutic benefits to treat bacterial infection. There are numerous approaches available for the simultaneous estimation of two medicines, but there is none accessible for the estimation of three medications, or the ones that exist are quite complicated, so the current study focuses on developing and validates a new, simple, rapid and novel spectrophotometric method for simultaneously estimating amoxicillin trihydrate, cloxacillin sodium, and probenecid in pure and solid dose forms using the simultaneous equation method. The λ_{max} of Amoxicillin Trihydrate (AMOX) (10 $\mu\text{g/ml}$), Cloxacillin Sodium (CLOX) (10 $\mu\text{g/ml}$), and Probenecid (PROB) (10 $\mu\text{g/ml}$) were 279.89 nm, 219.02 nm, and 239.67 nm, respectively. The medications followed Beer's rule and showed a good connection. Calibration curves for AMOX, CLOX, and PROB were linear from 5 to 30 $\mu\text{g/ml}$. The results indicated that the technique was accurate, precise, and reproducible (with a relative standard deviation of less than 1%) as per ICH guidelines. It is also affordable, simple to use, and time-saving, making it appropriate for the simultaneous determination of three medicines in commercial preparations. The proposed methods are recommended for routine analysis since they are rapid, simple, accurate, cost effective and also sensitive and specific. It involves neither heating nor use of any organic solvent for separation of the combination. Thus,

this study exploits the possibility for determining Pharmacokinetic data of the combined formulation which may be required in pre-clinical and clinical studies in near future.

Keywords Amoxicillin Trihydrate, Cloxacillin Sodium, Probenecid, Simultaneous Equation Method

1. Introduction

Chemically Amoxicillin Trihydrate is (2S,5R,6R)-6-[[[(2R)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid;trihydrate (C₁₆H₂₅N₃O₈S). It is broad spectrum antibiotics included in the penicillin group of the drugs. It acts as an antibacterial agent by inhibiting cell wall synthesis via inhibition of transpeptidase-c which helps in cross linking in the peptidoglycan linear structure. It is effective against gram-negative and gram-positive microorganism [1, 2].

Chemically Cloxacillin Sodium is (2S,5R,6R)-6-[[[3-(2-chlorophenyl)-5-methyl-1,2-oxazole-4-carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate;sodium (C₁₉H₁₇ClN₃NaO₅S). It is semisynthetic penicillin used as the salt form. Cloxacillin binds to the

penicillin binding protein found in bacteria's cell walls, inhibiting the final and third stages of cell wall formation of bacteria and providing antibacterial action. Cloxacillin is used to treat staphylococcal infection. Combined use of cloxacillin and amoxicillin is more effective to treat the bacterial infection [3].

Chemically Probenecid is 4-(dipropyl sulfamoyl) benzoic acid that acts as a uricosuric agent. Probenecid reduces serum urate levels while increasing uric acid excretion through urine by inhibiting tubular urate reabsorption. Combination of the three drugs is used as antibacterial agents. Probenecid decreases the renal tubular secretion of amoxicillin. Concurrent use of amoxicillin and probenecid may result in increased and prolonged blood levels of Amoxycillin [4].

Combined use of Amoxycillin trihydrate, cloxacillin trihydrate and probenecid is useful to treat the bacterial infections. Many studies are performed for simultaneous estimation of two drugs using UV spectrophotometric method.

Rojanarata *et al.* [5] reported a green bienzymatic UV-spectrophotometric assay of amoxicillin formulations. Beg *et al.* [6] investigated the pharmaceutical dosage form of amoxicillin trihydrate and determined that LC techniques give stability. Dangi *et al.* [7] used UV spectroscopy to develop a method for simultaneously estimating ranitidine bismuth and amoxicillin trihydrate. Kumar *et al.* [8] established a spectrophotometric technique for simultaneously estimating potassium clvulanate and amoxicillin trihydrate in a combined formulation. The simultaneous estimation technique for amoxicillin trihydrate and flucoxacillin sodium in capsule dosage forms was developed by Nikam DS *et al.* [9]. The simultaneous assessment of cloxacillin sodium and amoxicillin trihydrate by UV spectroscopy was reported by Solomon *et al.* [10].

According to a literature survey official and reported methods were found for the estimation of amoxicillin trihydrate, cloxacillin sodium and probenecid alone and with other combinations. There is no official or reported method available for the simultaneous estimation of amoxicillin trihydrate, cloxacillin sodium and probenecid drugs in the combined dosage form. The present work was directed towards the development of a UV-spectrophotometric simultaneous estimation of amoxicillin trihydrate, cloxacillin sodium and probenecid in their dosage form to validate the developed method as per ICH guideline.

2. Materials and Methods

2.1. Chemicals and Reagents

Working standards of amoxicillin trihydrate were procured from Cadila Pharmaceuticals Pvt Ltd Dholka.

Cloxacillin sodium was procured from Intas Pharmaceuticals. Probenecid was procured as a gift from Solitaire Pharma Ltd Methanol (AR grade), Sodium hydroxide (AR grade), Hydrochloric acid (AR grade), and 30 % Hydrogen peroxide (AR grade), which were used in the present study. Himox Plus tablets were procured from the market and used in the present study.

2.2. Apparatus

A UV-visible double beam spectrophotometer (Shimadzu 1800) was used for the experiment. An analytical Balance (systronic), Ultra Sonicator lab line 1-5L-50 was used.

2.3. Preparation of 0.1 N NaOH

It was prepared by dissolving 2 gm of sodium hydroxide (NaOH) in 500 ml distilled water.

2.4. Stock Solution Preparation

Standard dosages of Amoxicillin Trihydrate (AMOX), Cloxacillin Sodium (CLOX), and Probenecid (PROB) were precisely weighed at 10 mg each and transferred to individual 100 ml volumetric flask. The drug was then dissolved in 60 ml of 0.1 N Sodium hydroxide, the flasks were shaken well, and 0.1 N NaOH solutions were added to make 100 ml.

2.4.1. Selection of Detection Wavelength

To create a standard stock solution of 10 μ g/ml, an aliquot of the AMOX, CLOX, and PROB stock solution was diluted with 10 ml of methanol in a volumetric flask. These solutions were scanned between 200 and 400 nm to acquire spectra. AMOX showed significant absorbance at 279.89 nm, CLOX at 219.02 nm, and PROB at 239.67 nm. The overlap spectra of AMOX, CLOX and PROB are shown in Fig.1.

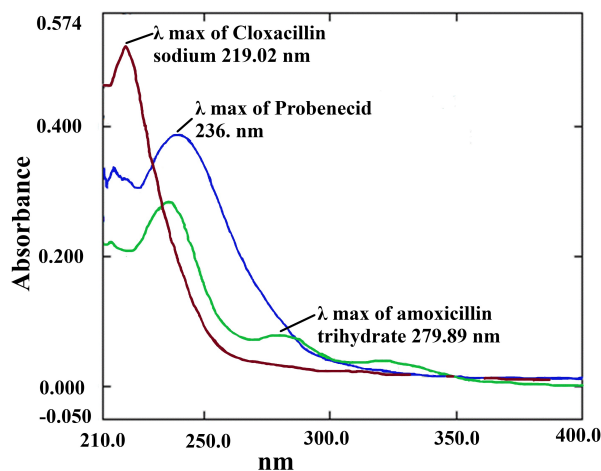


Figure 1. Overlay spectra of AMOX, CLOX and PROB

2.4.2. Preparation of Calibration Curve

A separate series of 10 ml volumetric flasks was filled with precisely measured standard stock solutions of AMOX, CLOX, and PROB (0.5, 1, 1.5, 2, 2.5, and 3 ml), which were then diluted to the appropriate level with 0.1 N NaOH. To generate the calibration curve, AMOX, CLOX, and PROB, 5, 10, 15, 20, 25, and 30 $\mu\text{g/ml}$ were utilized and estimated in UV-Visible spectrophotometer.

2.4.3. Simultaneous Equation Method

The Simultaneous Equation Mixture technique was used to compute the medicines' conc. A simultaneous equation may be able to identify the three absorbing medications (AMOX, CLOX, and PROB) present in the sample, each of which absorbs at the other's λ_{max} .

2.5. Analysis of Marketed Formulation

Himox Plus (amoxicillin trihydrate, cloxacillin sodium, probenecid each 250 mg).

2.5.1. Sample Solution Preparation

To determine the average weight of the tablets, a total of twenty Himox Plus tablets were taken and weighed. After being weighed, tablets become a fine powder. Powder weigh equal to 10 mg of amoxicillin trihydrate, cloxacillin sodium, and probenecid; transfer to a 100 ml volumetric flask; dissolve in 60 ml 0.1 N NaOH, flask shaken well and sonicated for 15 minutes; then use 0.1 N NaOH to adjust to the 100 ml mark. The sample solutions were filtered through whatman filter paper No. 41. The sample solutions were further diluted with 0.1 N NaOH to obtain 10 $\mu\text{g/ml}$ of Amoxicillin trihydrate, cloxacillin sodium, probenecid.

2.6. Validation of the Proposed Method

2.6.1. Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the amount of analyte in the sample. Linearity of the established method was achieved by running a series of standard mixtures of AMOX, CLOX and PROB. The standard calibration data were then calculated.

2.6.2. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. By doing the assay on the same day (intraday assay precision) and three other

days (interday precision), the reproducibility of the suggested procedure was ascertained. In order to conduct precision investigations, nine determinations of the procedure's defined range (3 x 3 duplicates for each concentration) were made. The method's good precision was evidenced by its low percentage RSD [10].

2.6.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. To calculate accuracy, the conventional addition approach was applied. To quantify sample solutions of AMOX, CLOX, and PROB (10 $\mu\text{g/ml}$), known amounts of standard solutions were added at 50%, 100%, and 150% levels. The percentage of recovery and the amount of drug recovered (mg) were computed [11].

2.6.4. Robustness

The robustness of the proposed methods was checked by changing the analyst and other conditions remained the same as UV spectrophotometer, solvent, dilutions, length.

2.6.5. Limit of Detection and Limit of Quantification

Each analytical process has a detection limit and a quantitation limit. The limit of detection is the lowest concentration of analyte in a sample that can be detected but not always quantified as an accurate number. While the lowest amount of analyte in a sample can be quantitatively determined with suitable precision and accuracy is called limit of quantification. A standard calibration curve served as the basis for determining the LOD and LOQ values. The LOD and LOQ can be determined using the residual standard deviation of the regression lines' y-intercept [10].

3. Results

NaOH (0.1 N) was used as the solvent for the AMOX, CLOX and PROB. The Overlap spectra of AMOX, CLOX and PROB (5-30 $\mu\text{g/ml}$) are shown in Figure 1. AMOX exhibits λ_{max} at 279.89 nm, CLOX exhibits λ_{max} at 219.02 nm and PROB exhibits λ_{max} at 239.67 nm. The calibration curve was linear over a concentration range of 5-30 $\mu\text{g/ml}$ for AMOX, CLOX and PROB. The developed simultaneous equation method was validated according to ICH guidelines. The optical regression characteristics and Validation parameters are presented in Table 1. % Recovery for AMOX, CLOX & PROB was found to be 99.492-101.696, 99.182-101.314, 98.82-103.73 respectively.

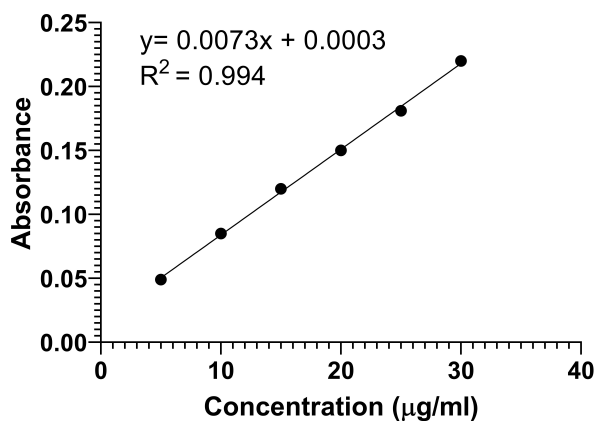
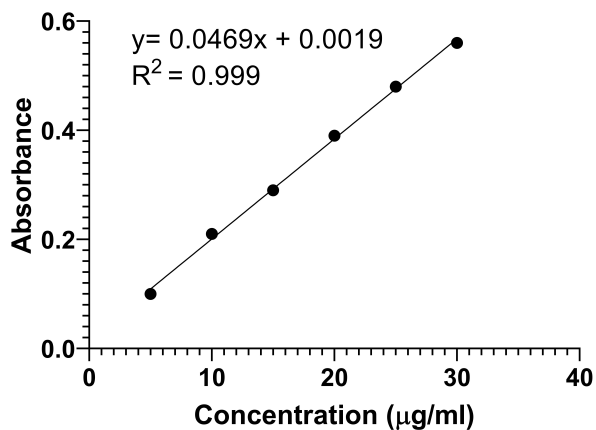
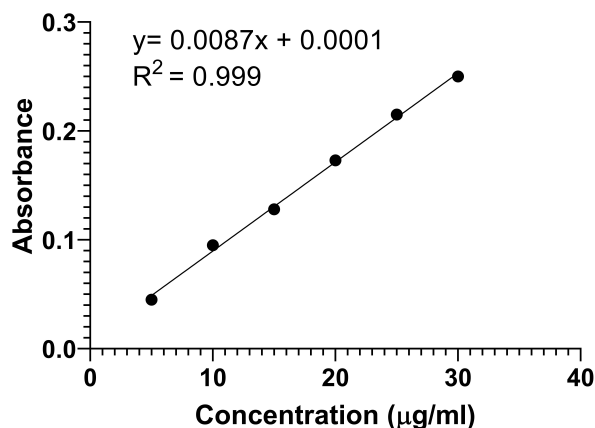
Table 1. Application to Synthetic Mixture

Synthetic mixture	Parameter	Amoxicillin trihydrate	Cloxacillin sodium	Probenecid
Mixture	Lable claim (mg/tablet)	250	250	250
	Amount found (n=3) (mg/tablet) Mean \pm S.D	257.66 \pm 1.222	247.7 \pm 0.271	246.96 \pm 1.341
	% drug content (n=3) Mean \pm S.D	99.08 \pm 1.32	99.28 \pm 1.28	98.78 \pm 2.30

3.1. Validation of the Analytical Method

3.1.1. Linearity

The linearity studies of this method were performed in the range of 5 to 30 $\mu\text{g/ml}$ of AMOX, CLOX and PROB. The linearity equations for AMOX, CLOX and PROB were found to be $y = 0.0073x + 0.0003$ with a correlation coefficient 0.994, $y = 0.0469x + 0.0019$ with a correlation coefficient 0.999, and $y = 0.0087x + 0.0001$ with a correlation coefficient 0.999 and also shown in Figs 2, 3 and 4, respectively.

**Figure 2.** Calibration curve of Amoxicillin trihydrate at 279.89 nm**Figure 3.** Calibration curve of Cloxacillin Sodium at 219.02 nm**Figure 4.** Calibration curve of Probenecid at 279.89 nm

3.1.2. Accuracy

Utilizing recovery studies, the suggested approaches' reliability and validity were evaluated. The method's sample recoveries are consistent with the claims made on each label, indicating that formulation additives do not interfere with the estimating process. Accuracy was performed at 50%, 100%, and 150 % levels by standard addition method. Data showed that at each level the % recovery of drug was 99 percentages which indicated that method was accurate for determination of drugs (Table 2).

3.1.3. Intraday Precision and Interday Precision

The precision under the same operating conditions over a brief period of time and intraday precision are indicated by the repeatability results. Calculations were made for the standard error, coefficient of variance, and SD. Six iterations of the tablet formulation were used to test repeatability. Table 3 presented the statistical evaluation results. By running the experiment on the same day (intra-day assay precision) and three separate days (inter-day assay precision), the reproducibility of the suggested procedure was ascertained. Tables 4 and 5 present the intra-day and inter-day precision results, which are expressed as a percentage of RSD.

3.2. Optical Regression Characteristics of Drugs

Table 6 represents the regression characteristics of AMOX, CLOX and PROB.

Table 2. Results of accuracy study (n=3)

DRUG	Level (%)	Amount taken (µg/ml)	Amount added (µg/ml)	Amount found ± S.D (µg/ml) (n=3)	RSD	% Recovery ± S.D (n=3)
Amoxicillin trihydrate (AMOX)	-	10	0	9.98±0.15	1.55	99.88±1.55
	50	10	5	15.25±0.11	0.73	101.69±1.11
	100	10	10	19.89±0.18	0.92	99.49±1.83
	150	10	15	25.16±0.13	0.53	100.64±1.34
Cloxacillin sodium (CLOX)	-	10	0	9.89±0.05	0.53	98.91±0.52
	50	10	5	15.19±0.03	0.24	101.31±0.37
	100	10	10	19.92±0.04	0.24	99.60±0.48
	150	10	15	24.79±0.07	0.30	99.18±0.76
Probenecid (PROB)	-	10	0	10.77±10.17	1.16	100.72±1.17
	50	10	5	14.82±0.18	1.22	98.82±1.81
	100	10	10	20.37 ±0.19	0.96	101.86±1.96
	150	10	15	25.93±0.13	0.51	103.73±1.33

Table 3. Results of precision data of AMOX, CLOX and PROB

Drug	Concentration (µg/ml)	Absorbance at 279.89 nm	Absorbance at 219.02 nm	Absorbance at 239.67 nm
Amoxicillin trihydrate (AMOX)	25	Mean ± S.D =0.1745±0.0034	Mean ± S.D =0.4795±0.0043	Mean ± S.D =0.6736±0.007
		% RSD =1.9782	% RSD =0.9150	% RSD =1.0449
Cloxacillin sodium (CLOX)	25	Mean ± S.D =0.0455±0.0018	Mean ± S.D =1.1806±0.017	Mean ± S.D =0.44±0.0052
		% RSD =1.67	% RSD =1.45	% RSD =1.20
Probenecid (PROB)	25	Mean ± S.D =0.223±0.0044	Mean ± S.D =0.848±0.0066	Mean ± S.D =0.95±0.0069
		%RSD =1.99	%RSD =0.77	%RSD =0.73

Table 4. Precision study (Intraday precision)

Conc. (µg/ml)	Intraday Mean ± S.D (n=3)			% RSD		
	219.02 nm	239.67 nm	279.89 nm	219.02 nm	239.67 nm	279.89 nm
10	0.181±0.0011	1.007±0.0092	0.816±0.0066	0.62	0.92	0.82
15	0.272±0.0013	1.501±0.0167	1.224±0.0097	0.5	1.11	0.79
20	0.363±0.0030	2.001±0.0144	1.632±0.0099	0.85	0.72	0.61

Table 5. Precision study (Interday precision)

Conc. (µg/ml)	Inter day Mean ± S.D (n=3)			%RSD		
	219.02 nm	239.67 nm	279.89 nm	219.02 nm	239.67 nm	279.89 nm
10	0.201±0.0012	1.023±0.0115	0.923±0.0176	0.61	1.13	1.90
15	0.342±0.0039	1.612±0.0195	1.341±0.0183	1.15	1.21	1.36
20	0.421±0.0050	2.023±0.0309	1.723±0.0198	1.19	1.52	1.15

Table 6. Optical regression characteristics AMOX, CLOX, PROB

Parameters	Values		
	AMOX	CLOX	PROB
Wavelength (nm)	279.89	219.02	239.67
Beer's Law Limit ($\mu\text{g/ml}$)	5-30	5-30	5-30
Regression Equation ($y = mx + c$)	$Y=0.0073X+0.0003$	$Y=0.0469X+0.0019$	$Y=0.0368X+0.0079$
Slope (m)	0.0073	0.0469	0.0368
Intercept (c)	0.0003	0.0019	0.0079
Correlation Coefficient (r)	0.994	0.999	0.998
LOD ($\mu\text{g/ml}$)	0.557	0.463	0.334
LOQ ($\mu\text{g/ml}$)	1.158	1.390	1.000

4. Discussion

AMOX, CLOX, and PROB were calculated using a simultaneous equation technique. The highest wavelengths (λ) of Amoxicillin Trihydrate (10 $\mu\text{g/ml}$) were 279.89 nm, Cloxacillin Sodium (10 $\mu\text{g/ml}$) at 219.02 nm, and Probenecid (10 $\mu\text{g/ml}$) at 239.67 nm. The linearity of this approach ranged from 5 to 30 $\mu\text{g/ml}$. The correlation coefficient, intercept, and slope for AMOX were 0.994, 0.0003, and 0.0073, respectively; for CLOX, 0.999, 0.0019, and 0.0469, respectively; and for PROB, 0.998, 0.0079, and 0.0368, respectively. The amount of drugs estimated and the percentage estimation were computed using the equation of the standard mixture and synthetic mixture that were analyzed using the simultaneous equation approach. It was discovered to be within the allowed range. In accordance with ICH criteria, the suggested method's linearity, precision, accuracy, LOD, and LOQ were validated. The accuracy investigation was carried out at three different recovery levels: 98.88-101.69 percentage AMOX, 98.91-101.31 percentage CLOX, and 98.82-103.73 percentage PROB. The method's great accuracy and precision may be shown by the standard deviation and percent RSD value, both of which were found to be less than 2% showing the high precision and accuracy of the method. By varying the analyst, the suggested method's robustness was calculated and determined to be within a range. It was discovered that the quantification and detection limits for AMOX, CLOX, and PROB were, respectively, 0.334 and 1 $\mu\text{g/ml}$, 0.463 and 1.390 $\mu\text{g/ml}$, and 0.557 and 1.158 $\mu\text{g/ml}$. Numerous strategies are available for the study of medicines in multi-component formulations using this spectroscopic method. A study of a typical mixture and formulation incorporating three medications revealed that the approach was straightforward, exact, accurate, and repeatable.

5. Conclusions

This technique offers a quick analytical process for

estimating amoxicillin trihydrate, probenecid, and cloxacillin sodium all at once. The method's accuracy and reproducibility were indicated by the satisfactory low standard deviation. The acceptable results of recovery studies indicate that excipient interference was not present. Quality control can benefit from the application of these methods due to their low quantification and detection limits. It was discovered that the created method is easy to use, quick to execute, and accurate for routinely estimating three different medications from tablet formulations. It involves neither heating nor use of any organic solvent for separation of the combination. Thus the study exploits the possibility for determining Pharmacokinetic data of the combined formulation which may be required in pre-clinical and clinical studies in near future.

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Nil.

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