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Research Article

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Validated HPTLC method for determination of dicloxacillin in simulated urine

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ABSTRACT

A simple, selective, and sensitive HPTLC method for the determination of Dicloxacillin in Simulated Urine was developed & Validated. The method utilizes Protein precipitation as the sample preparation technique. The HPTLC separation was achieved on the aluminum backed layer of silica gel 60 F_{254} using Toluene : methanol : Triethylamine (4:6:0.5% v/v/v) as mobile phase. The Detection wavelength used was 227nm. The chamber saturation time is 15min.Cefixime was used as an internal standard. The calibration curve was linear (r^2>0.99) through the range of 200-1000ng/band. The mean recovery was found to be 93.31% for Dicloxacillin. Quantification was achieved with HPTLC, over the concentration range of 200 to 1000 ng/band. Retention factor (Rf) for Dicloxacillin and Cefixime (IS) were 0.80 and 0.50 respectively. Dicloxacillin was found to be stable in simulated urine. The method showed acceptable values for accuracy, precision, recovery and stability.

Keywords: HPTLC, simulated Urine matrix, Dicloxacillin, Cefixime, Protein Precipitation.

INTRODUCTION

Dicloxacillin sodium [1,2] ,:(2*S*,5*R*,6*R*)-6-{[3-(2,6-dichlorophenyl)-5-methyl-oxazole-4-carbonyl] amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid (DICLO) is oral penicillinase resistant penicillin. It has isoxazolyl side chain that protects the β-lactum ring from the attack of *staphylococcal* penicillinase. It is official in USP[3], BP[4].

Dicloxacillin sodium (DICLO) alone and in combination with other drugs in pharmaceutical preparations and in plasma was Detected & Quantified by HPLC, LC-MS methods[5-7]. No references have been found for quantitative determination of DICLO using Urine as a biological matrix. As > 50% DICLO is excreted unchanged in urine, here attempts were made to develop HPTLC method for determination of DICLO in simulated urine matrix [8]. Cefixime (CEF) was used as a Internal standard.

In this paper we report simple, accurate, precise and sensitive high performance thin layer chromatography method for quntitative determination of DICLO in urine matrix. The structure for DICLO is described in Figure 1. The method has been validated as per the US CDER (May 2001) Guidelines. [9,10]

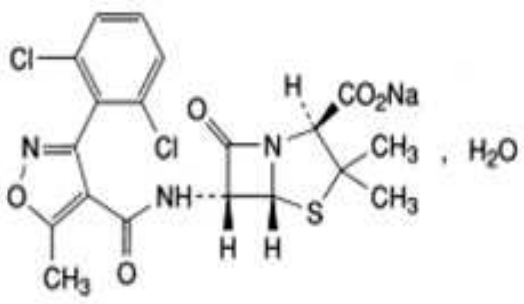

Fig 1: **Structure of Dicloxacilin sodium(DICLO)**

EXPERIMENTAL SECTION

Drugs, Reagents and Chemicals used

Dicloxacilin and Cefixime were kindly provided by Maxim Phramaceuticals, Pune, India as gift samples. Methanol AR Grade was purchased from SD Fine Chemicals Laboratories, Mumbai. Toluene AR grade was purchased from Loba Chemicals, Triethylamine AR Grade was purchased from Rasayan chemicals. Chromatographic separation was performed on aluminium plates pre-coated with silica gel 60 F254, purchased from E-Merck, Germany. Samples were applied on the plate as a band with 4 mm width using Camag 100 µL sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (10 x 10 cm) at room temperature and a densitometry scanning was performed using Camag TLC scanner 3 in the range of 400-2000 nm, operated by winCATS software (Version 1.4.3, Camag). Deuterium lamp was used as a radiation source. All weighing was done on Shimadzu balance (Model AY-120).

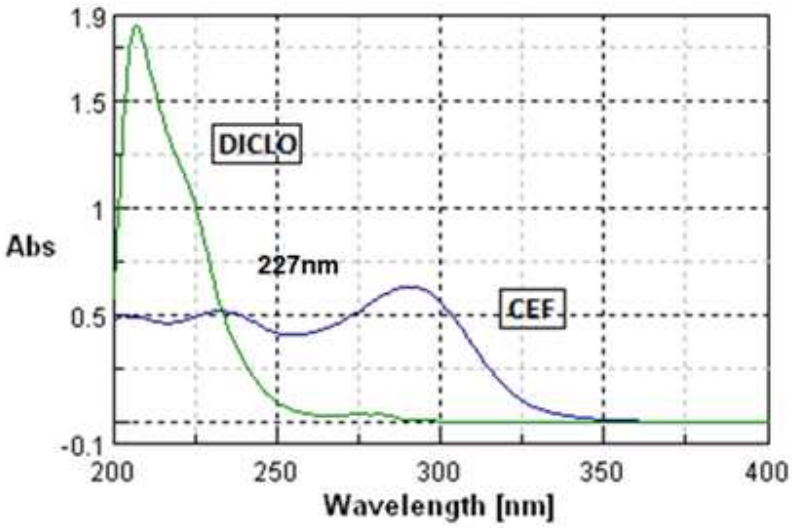

Figure 2: Overlain spectra of DICLO (100µg/ml) & CEF(10µg/ml)

Preparation of mobile phase and stock solutions:

AR grade Toluene, Methanol & Triethylamine was mixed with in 4:6:0.5 v/v/v proportion. Stock solution was prepared by dissolving 10 mg DICLO in 10ml methanol to get concentration of 1000 µg/ml. Working stock solution for DICLO was prepared by diluting appropriately stock solution to get the final concentrations of 100µg/ml. Solution of internal standard was prepared by dissolving 10mg in 10ml methanol to get concentration of 1000 µg/ml. 1ml of this solution was diluted to 10ml with methanol to get the final concentration of 100 µg/ml.

Checking The Resolution of Drugs

100 µg/ml of each of DICLO and CEF were prepared as stock solutions in methanol as described. 4µL of DICLO solution(400ng/spot) was spotted on the plate using 100 µl Hamilton syringe and 4µL CEF (400ng/spot) was co spotted on the DICLO spot. The development chamber was saturated with mobile phase for 15 min. The spotted plate was placed in the saturated chamber and developed up to 90 mm distance. The plate was dried and scanned over 90 mm distance. The retention factors of DICLO and CEF were at Rf of 0.80 ± 0.05 and 0.50 ± 0.05 respectively.

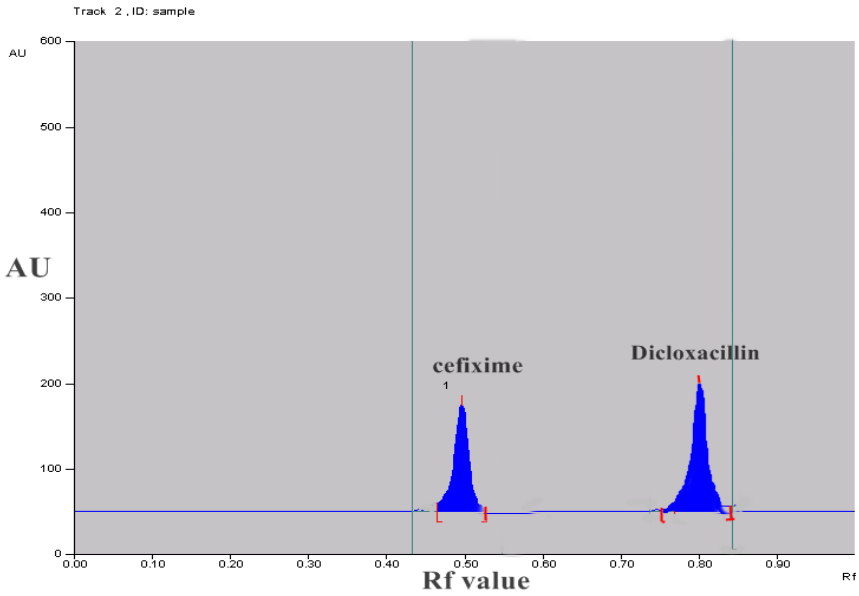

Figure 3 : Densitogram of CEF and DICLO

Table 1: Summary of chromatographic parameters

Sr. no.	*Parameters*	*Optimized conditions*
1.	Stationary phase	Aluminium plates Precoated silica gel 60 F_{254}
2.	Detection wavelength	227 nm
3.	Mobile phase	Toluene : Methanol : Triethylamine (4:6:0.5 v/v/v)
4.	Saturation time	15 min

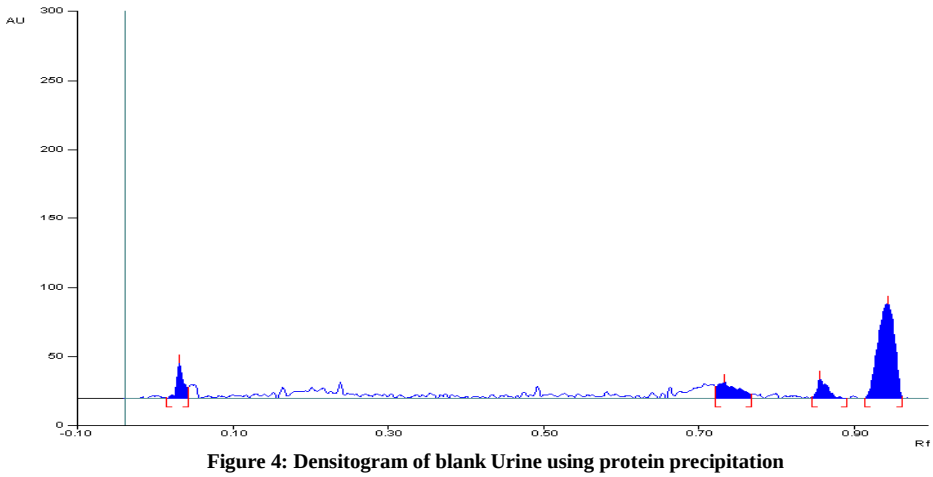

Figure 4: Densitogram of blank Urine using protein precipitation

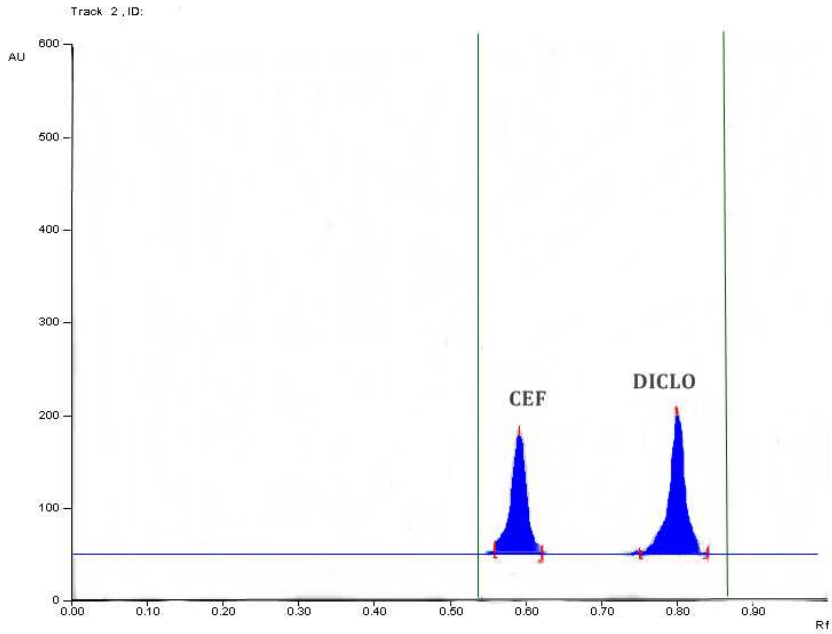

Figure 5: Densitogram of Urine spiked with DICLO (400 ng/band)(Rf-0.80 ± 0.05) and CEF (IS) (400 ng/band)(Rf-0.50 ± 0.05

Preparation of simulated urine

The Urine matrix has been prepared as per following formula as [8],

Table 2: Composition of Simulated Urine sample

Sr no	Composition	Amount (mg//100ml)
1	Uric acid	16.81mg
2	Creatinine	52.45mg
3	Sodium chloride	315.5mg
4	Potassium chloride	223.6mg
5	Calcium chloride	33.29mg
6	Sodium bicarbonate	16.8mg
7	Sodium biphosphate	59.01mg
8	Magnesium sulphate	24.07mg
9	Ammonium chloride	80.23mg
10	Urea	120.12mg
11	Water	Qs

Sample preparation

We have performed our method development & validation using Simulated Urine as a matrix therefore, sample pretreatment may not be necessary as far as method validation parameters are concerned. When method is applied to Clinical sample, Protein Precipitation is required to be done for sample preparation.

METHOD VALIDATION

The method was validated as per US CDER guidelines for Selectivity, linearity, intraday and interday precision, accuracy, extraction, recovery and stability.

Selectivity

The analytical method should be able to differentiate the analyte(s) of interest and IS from endogenous components in the matrix or other components in the sample. The selectivity of the method was evaluated by analyzing Simulated Urine samples spiked at LLOQ (Lower Limit of Quantification) i.e. 200 ng/band).

Calibration curve
Standard stock solution was prepared by dissolving 10 mg of DICLO and 10mg of CEF in 10 ml methanol (1000 µg/ml) separately. From stock solution of each of DICLO and CEF, 1ml was diluted to 10 ml with methanol to get 100µg/ml concentration.

Linearity was tested for the range of concentrations 200-1000ng/band for HPTLC, similarly calibration curve was checked in simulated urine in the same concentration range. Each sample in five replicates was analyzed and peak areas were recorded. The response factor for each concentration was calculated by taking ratio of peak area of Dicloxacillin and IS. The response factors were then plotted against the corresponding concentrations to obtain the calibration graphs.

Accuracy, precision and lower limit of quantification:
The accuracy and precision of the method were evaluated using the Quality Control samples. Intra-day accuracy and precision was measured by consecutively analyzing Q.C. samples in simulated urine in one single day. The procedure was repeated for three different days to test the inter-day accuracy and precision. Accuracy was calculated as percentage accuracy, whereas precision was measured in terms of coefficient of variation (C.V.) of each calculated concentration. Lower limit of quantification (LLOQ) was found to be 200 ng/band for HPTLC, since the response obtained was five times the response compared to blank.

Recovery:
Recovery for Dicloxacillin was evaluated at three concentration levels corresponding to three routine Q.C. samples 200,600,1000 ng/band for HPTLC analyzed in triplicate. Recovery was determined by comparing the ratio of the peak area of Dicloxacillin obtained after the application of the processed urine calibration samples with those achieved by working standard solution in the methanol.

Stability:
As per US CDER guidelines, stability was checked under different conditions viz.
Freeze- thaw stability
Short term stability
long term stability
Stock solution stability &
Post preparative stability

Freeze-thaw stability of Dicloxacillin was determined by assaying low and high Q.C. samples for HPTLC 200, 1000 ng/band in triplicate over three freeze-thaw cycles. First freeze-thaw cycle consisted of 24 hrs freezing at -5^0C followed by a complete thaw at a room temperature (RT). The next two freeze-thaw cycles were of 12 hrs each frozen state at -5^0C followed by a complete thaw at a room temperature. Short term stability consisted of two Q.C. samples stored for 4 hrs at room temperature and long term stability involved storage of two Q.C. samples for 14 days at 4^0 C. For stock solution stability, the stock solutions of the drug and IS were stored for period of 5 days in refrigerator at 4^0 C and then for 6 hrs at room temperature. Post preparative stability, where stability of the spiked samples for MQC of Dicloxacillin and IS were determined after the storage for 5 hrs at room temperature. All these Q.C. samples were then evaluated in triplicate and the results were compared with the freshly prepared samples of same concentrations.

RESULTS AND DISCUSSION

Retention Factor (R_f) for Dicloxacillin and IS were 0.80 and 0.50 respectively. As previously stated the representative Densitograms of CEF & DICLO, blank urine and urine spiked with Dicloxacillin (200ng/band) are shown in Figure 3, 4 and 5 respectively.

Selectivity:
The selectivity of the method was evaluated by analyzing simulated urine samples spiked at LLOQ 200ng/band for HPTLC in which no interference by endogenous components was noted. % CV (Coefficient of variation) for 6 replicates spiked at LLOQ was found to be 1.00 %

Calibration curve:
Calibration curve was constructed by plotting Response factor Vs concentration of Dicloxacillin solutions, and the regression equation was calculated. The calibration curve was plotted over the concentration range 200-1000ng/band. With correlation coefficient 0.995, a mean slope of 0.003, mean y-intercept of 0.346.

Accuracy:
The method showed good accuracy and precision in plasma samples. Table 3 shows the results for intra- and inter-day precision and accuracy for Dicloxacillin in urine samples. Mean % accuracy of all quality control samples was found to be 98.74 ± 0.33 .(LLOQ was found to be 200ng/band)

Table 3 : Accuracy Of Dicloxacillin simulated urine QC Samples

Theoretical Conc. (ng/band)	Observed Conc.(mean ng/band ± SD)	Precision (%C.V.)	Accuracy (%)
200	197.8 ±1.02	0.91	98.93
600	593.1 ± 7.41	0.48	98.86
1000	988.4 ± 12.02	0.79	98.44
Average		0.72 ± 0.22	98.74 ± 0.33

Recovery:
Table 4 shows the results of the recovery tests for the three Q.C. levels tested (200,600 and 1000 ng/band). The recovery in urine samples ranged from 90.17 to 96.50 % for Dicloxacillin at three concentration levels. The mean recovery for Dicloxacillin was found to be 93.30 %.

Table 4 : Recovery Of Dicloxacillin In Simulated Urine Q.C. Samples

QC Levels(ng/band)	% C.V.	Recovery (%)
200	0.67	93.25
600	0.38	90.17
1000	0.26	96.50
Average	0.43	93.30

Stability Studies:
Simulated urine Q.C. samples at two concentrations (200 and 1000 ng/band) was used for freeze-thaw, Short term and long term stability studies. Stock solution stability was performed at three concentrations (200,600,1000 ng/band).Post preparative stability was performed for the drug (600 ng/band) and IS (400ng/band). It was performed to evaluate the influence of storage conditions from the sample collection to analysis. Table 5 represents the results of stability studies. Results indicated that Dicloxacillin is stable in simulated urine for the given stability conditions. The coefficient of variation(CV) of the mean test responses to the freshly prepared solutions was less than 15% at any of the stability conditions.

Table 5: Stability Of Dicloxacillin In Simulated Urine Q.C. Samples

Stability	Conc. (ng/band)	Mean Stability (%)	% C.V.
Freeze Thaw Stability	200	96.90	0.68
	1000	97.33	0.17
Short term stability	200	96.91	1.36
	1000	97.32	0.11
Long term stability	200	93.13	1.12
	1000	97.30	0.10
Stock solution stability	200	94.79	0.80
	600	97.63	0.22
	1000	97.37	0.07
Post preparative stability	600	92.04	0.24
	400 (IS)	94.36	0.66

CONCLUSION

Most published methods to quantify Dicloxacilin in body fluids use tedious extraction, purification steps and sometimes solid phase extraction or some other tedious procedures have been applied to get rid of interfering proteins and other matter from the selected matrix. In this study, rapid and sensitive HPTLC method has been

developed for the determination of Dicloxacillin in Urine by Protein Precipitation extraction technique which is with simple and limited steps. Validation results proved that the developed method performs well with selectivity, precision, accuracy, stability and linearity for the concentration range of Dicloxacillin expected to be found in urine after oral administration of 250-500mg dose. The validated method covers the range of linearity over 200-1000 ng/band and is therefore suitable for the determination of Dicloxacillin in Urine at different therapeutic dose levels. The mean recovery of Dicloxacillin is 93.31%. The resolution between Dicloxacillin and endogenous substances was satisfactory. The proposed method can be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis. The developed method is able to measure concentration of Dicloxacilin which can be used in Urine for dose regulation and bioavailability studies.

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