International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.2, pp 407-413, April-June 2013

Detemination Of Dicloxacillin In Human Plasma By HPLC Method

Rupali B. Harale[1], Madhura V. Dhoka[1]*, Mrinalini C. Damle[1]

[1]Department of Quality Assurance, AISSMS College of Pharmacy, Near R.T.O, Kennedy Road, Pune - 411001.India.

***Corres.author: madhura1777@yahoo.com**
Tel. No: - +91-9422079037

Abstract: Dicloxacillin is an oral penicillinase resistant penicillin. It has isoxazolyl side chain that protects the β-lactum ring from the attack of penicillinase producing bacteria. Rapid, simple, selective, and sensitive high performance liquid chromatography method for the determination of Dicloxacillin in human plasma has been developed. Acetanilide was used as an internal standard (IS). The method utilizes simple protein precipitation as the sample preparation technique using acetonitrile as a precipitating agent. HPLC method was developed using HiQ sil C18 HS column, with the mobile phase containing mixture of methanol: phosphate buffer 0.01M (pH 3.0, adjusted with glacial aceteic acid) (70: 30% v/v), at the flow rate of 1ml/min and detection was performed at 227nm. Retention times for Dicloxacillin (DICLO) and the internal standard (IS) were 7.7 and 4.6 min, respectively. The calibration curve was linear (r^2>0.99) through the range of 10-18 µg/ml. The lower limit of quantification was found to be 10 µg/ml. % R.S.D. was less than 5% for intra- and inter-day precision. The mean recovery was found to be 98.98% for Dicloxacillin. Method was validated as per US CDER guidelines. The method showed acceptable values for accuracy, precision, recovery, sensitivity and stability. The method is well suited for routine analysis of Dicloxacillin in human plasma and can further be extended for pharmacokinetic studies.

Keywords: HPLC, human plasma, Dicloxacillin, protein precipitation.

Introduction:

Dicloxacillin sodium, 4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid, 6-[[[3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolyl] carbonyl] amino]-3,3-dimethyl-7-oxo-, monosodium salt, monohydrate, is oral penicillinase resistant penicillin. It has isoxazolyl side chain that protects the β-lactum ring from the attack of penicillinase producing bacteria[1]. It is official in USP 30 and BP 2007[(2-3)].

Dicloxacillin is better absorbed from the gastrointestinal tract than cloxacillin but absorption is reduced by the presence of food in the stomach. After an oral dose of 500 mg, peak plasma concentrations of 10 to 18 µg/ml in about 1 hour have been reported in fasting subjects. Dicloxacillin has been reported to have a plasma half-life of 0.5 to 1 hour. Dicloxacillin is metabolised to a limited extent and the unchanged drug and metabolites are excreted in the urine by glomerular filtration and renal tubular secretion. About 60% of an oral dose is excreted in the urine.

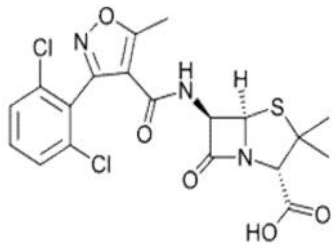


Figure 1. Structure of Dicloxacillin

There are few methods have been reported for the determination of Dicloxacillin in human plasma[4]. However, the methods are expensive and therefore may not be employed for routine analysis. Reported developed methods suffer from drawbacks viz.

i) Tedious, complicated extraction procedure which includes evaporation to dryness under nitrogen steam.

ii) Many additional washing steps are required so more time is required for plasma sample preparation.

The present method describes a simple, selective, and sensitive HPLC method with UV detection with a calibration range of 10-18 µg/ml for Dicloxacillin in human plasma. The method utilizes simple, rapid protein precipitation technique with acetonitrile as the sample preparation technique. Acetanilide was used as an internal standard (IS). The structures for Dicloxacillin and IS are described in Figure 1.The mobile phase consist of Methanol: Phosphate buffer (0.01M) pH 3 (70:30v/v) which was easy to prepare. The method has been validated as per the Guidelines for US CDER [5].

Materials And Methods

Instruments

Chromatographic separation was performed on a Jasco chromatographic system equipped with a Jasco HPLC pump Model PU2080 plus, Jasco UV-2075 plus detector, Rheodyne injector with 50µl loop volume and Elga water system for HPLC grade water.

Chemical s and Reagent:

Dicloxacillin working standard and acetanilide (IS) was kindly supplied by Maxim Pharmaceuticals, Pune. AR grade methanol, dihydrogen phosphate, acetonitrile, glacial aceteic acid were purchased from S. D.fine-chemical Laboratories, Mumbai, India.

General procedure:

The chromatographic parameters are given below

Chromatographic conditions-

Mobile Phase- Methanol: Phosphate buffer 0.01M (pH-3.0 adjusted with gacial aceitic acid)
(70:30 v/v)
Flow rate -1.0 mL min^{-1}
Injection volume- 50 µL
Detection wavelength -227 nm
Column- HiQ sil C18 HS column
Internal Standard- Acetanilide

Sample preparation-

i) Working standard solution.

10 mg of Dicloxacillin was dissolved in 10 mL of methanol to obtain 1000 µg/mL of standard solution. From this solution 1 mL was taken and made the volume to 10 mL so as to obtain the concentrations of 100µg/mL.

From this aliquots of 4, 4.8, 5.6, 6.4, 7.2ml were diluted to 10 ml so as to obtain the concentrations of 40, 48, 56, 64, 72 µg/ml as working stock solutions, which were used for preparing the plasma samples.

For internal standard, 10 mg of Acetinilade was dissolved in 10 ml of methanol to obtain 1000 µg/ml. From this 0.2 ml was diluted to 10 ml with methanol to obtain concentration of 20 µg/ml.

ii) Plasma Sample preparation.

For Plasma sample preparation, from each of above working standard solution 0.5ml was taken and added to 0.5ml of plasma. To this solution 0.5ml of Acetonitrile was added to precipitate proteins. The contents of the tubes were vortexed for 2 min. To this 0.5 ml stock solution of IS (20 µg/ml) was added and vortexed for 2 min. It was centrifuged for 10 minutes at 2500 rpm. The final plasma solutions were obtained in concentrations of 10, 12, 14, 16, 18 µg/mL. 50µl was injected from the above solution in the stabilized chromatographic system.

The calibration curve for Dicloxacillin was obtained using five calibration standard levels (10, 12, 14, 16, 18 µg/ml). Linear regression analysis was done, considering the ratio of the peak area of analyte to internal standard versus concentration applied. A correlation coefficient of more than 0.99 was obtained for calibration curve.

Valiadation of the method

The method was validated as per *US CDER* guidelines.

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from different sources spiked at LLOQ (Lower Limit of Quantification).

Calibration/standard curve

Linearity was tested for the range of concentrations 10-18µg/ml. 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 precision of the method were evaluated using the Q.C. samples. Intra-day precision was measured by consecutively analyzing Q.C. samples in one single day. The procedure was repeated for three different days to test the inter-day precision. Accuracy was evaluated using five replicate of LLOQ, LQC, MQC, HQC samples. Accuracy was calculated as percentage accuracy whereas precision was measured in terms of relative standard precision (R.S.D.) of each calculated concentration. The Lower limit of quantification was decided on the basis of lowest concentration on calibration curve.

Recovery

Recovery for *Dicloxacillin* was evaluated at three concentration levels corresponding to three Q.C. samples (LQC, MQC and HQC) 12, 14, 16µg/ml analyzed in triplicate for HPLC. The % mean recovery of Dicloxacillin was determined by measuring the responses of the extracted plasma quality control samples against unextracted quality control samples at LQC, MQC and HQC levels.

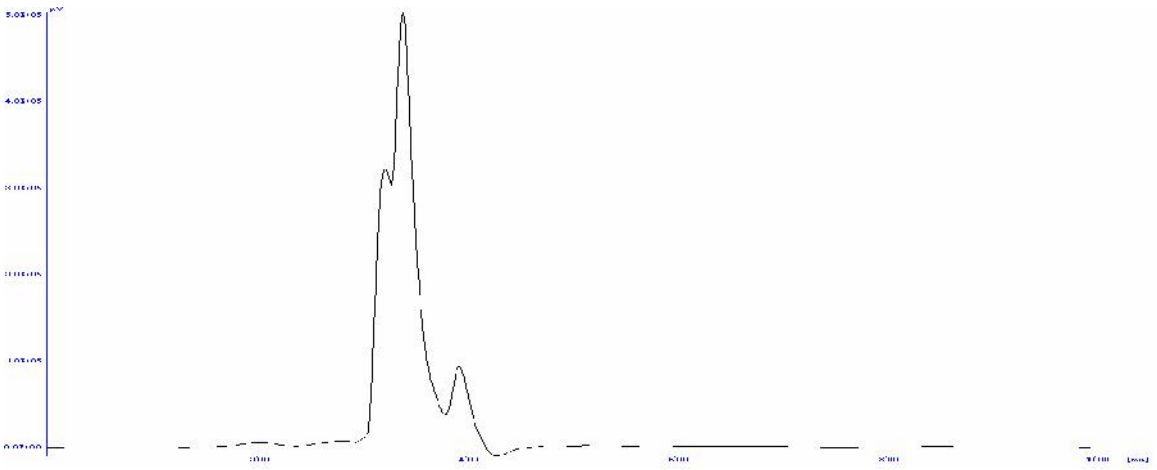
Stability

As per US CDER guidelines, stability was checked under different conditions viz.

1. Freeze- thaw stability
2. Short term stability
3. long term stability
4. Stock solution stability
5. Post preparative stability

Freeze-thaw stability of *Dicloxacillin* was determined by assaying low and high Q.C. samples that is 12, 16 µg/ml in triplicate over three freeze-thaw cycles. First freeze-thaw cycle consisted of 24 hrs freezing at -5^0 C followed by a complete thaw at a room temperature. The next two freeze-thaw cycles were of 12 hrs each frozen state at -5^0 C followed by a complete thaw at a room temperature.

Short term stability was determined by analysing of two Q.C.(LQC & HQC) samples stored for 4 hrs at room temperature and long term stability involved storage of two (LQC & HQC) 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.



"Figure 2: Typical chromatogram of blank human plasma"

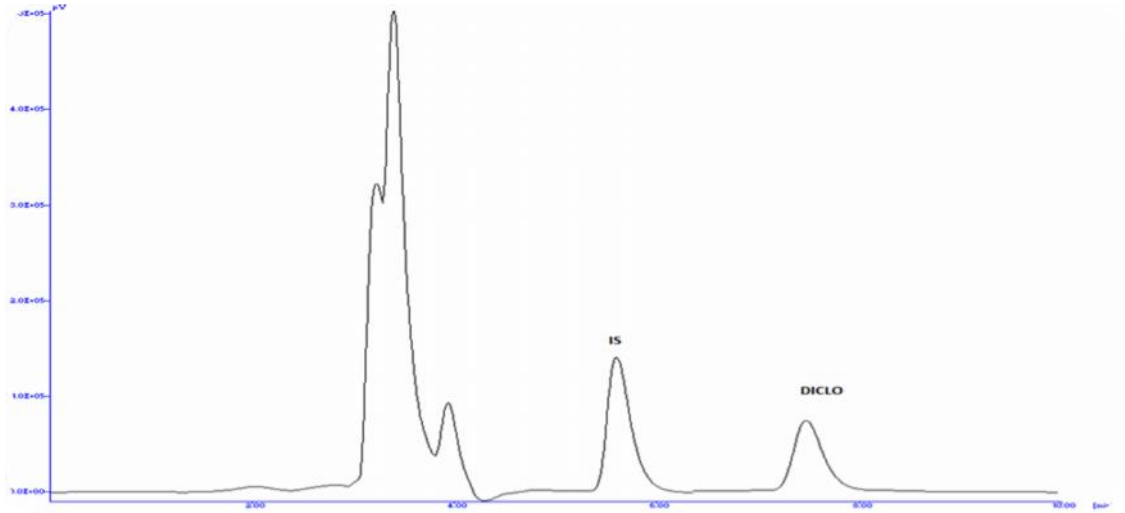


Figure 3: Typical chromatogram of blank human plasma spiked with IS, 20µg/ml (Rt-4.6 min.) and DICLO, 10µg/ml (Rt-7.7 min.)

Results:

Chromatographic characteristics

A mobile phase consisted of Methanol: Phosphate buffer 0.01M (pH-3.0 adjusted with gacial aceitic acid) (70:30 v/v) offered a good separation at flow rate 1ml/min and at run time of 7.7min for DICLO and 4.6 min for IS as shown in Fig 3.

Selectivity

The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from different sources spiked at LLOQ (10µg/ml) in which no interference by endogenous components was noted. % RSD (Relative standard deviation) for 6 replicates spiked at LLOQ was found to be 1.76%.

Calibration/standard curve

The data for linearity studies was found to be best fitted by linear equation y = mx + c in the range of concentration 10- 18µg/ml. With correlation coefficient 0.999, a mean slope of 0.017, mean y-intercept of 0.190

Accuracy, precision and lower limit of quantification

The method showed good accuracy and precision in plasma samples for LQC, MQC and HQC Table 1 shows the results for intra- and inter-day precision and Table 2 shows the results for accuracy of *Dicloxacillin* in plasma samples. %CV for intraday precision 3.12 ± 1.20 and for inter-day precision 2.78 ± 0.40 respectively. The % mean accuracy for all quality control samples at LQC, MQC and HQC concentration levels were in the range of 95.41 to 98.76, LLOQ was found to be 10µg/ml

Table 1: Intra-day, inter-day precision of in human plasma QC samples.

Theoretical (µg/ml)	Observed mean (µg/ml ± SD)	Precision (%CV)
Intra-day		
12	11.1 ±0.447	4.028
14	13.14 ± 0.230	1.752
16	15.06 ± 0.541	3.594
Average		3.124 ±1.20
Inter-day		
12	11.22± 0.249	2.80
14	13.05 ± 0.15	3.18
16	15.38 ± 0.219	2.37
Average		2.78 ± 0.40

Table 2: Accuracy of in human plasma QC samples

Theoretical Conc. (µg/ml)	Observed Conc.(mean µg/ml ± SD)	Accuracy (%)
12	12.03 0.39	100.27
14	13.35 0.25	95.41
16	15.80 0.89	98.76
Average		98.14 ± 2.48

Recovery

Table 3 shows the results of the recovery tests for LQC, MQC and HQC levels. That is 12, 14, and 16 µg/ml. The extraction recovery in plasma samples ranged from 96.42 to 100.97 % for Dicloxacillin at three concentration levels. The mean recovery for Dicloxacillin was found to be 98.98 %.

Stability

It was performed to evaluate the influence of storage conditions from the sample collection to analysis. Table 4 represents the results of stability studies. Results indicated that *Dicloxacillin* is stable in human plasma for the given stability conditions.

Table 3: Recovery of Dicloxacillin in human plasma Q.C.samples.

QC Levels(µg/ml)	% CV	Recovery (%)
12	0.17	99.53
14	2.45	96.42
16	5.03	100.97
Average	2.55	98.98 ± 2.32

% CV. = SD/mean x 100

Table 4: Stability of Dicloxacillin in human plasma Q.C. samples.

Stability	Conc. (µg/ml)	Mean Stability (%)	% CV
Freeze thaw stability (three cycles)	12	99.40	2.13
	16	101.33	1.88
Short term stability (for 4h at RT)	12	96.82	4.57
	16	99.36	3.45
Long term stability (for 14 days at 4^0 C)	12	97.31	4.15
	16	98.73	2.09
Stock solution stability (for 5 days at 4^0 C, 6hrs at RT)	14	98.92	2.94
	20(IS)	97.51	0.90
Post preparative stability (for 5hrs RT)	14	97.28	2.73
	20(IS)	97.90	1.46

% CV = SD/mean x 100, RT (room temperature)

Discussion:

Most published methods to quantify Dicloxacillin in body fluids use tedious extraction and many purification steps. In this study, rapid and sensitive HPLC method has been developed for the determination of Dicloxacillin in human plasma by simple protein precipitation extraction technique. 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 human plasma after oral administration of 500mg dose. The validated method covers the wide range of linearity over 10-18 µg/ml and is therefore suitable for the determination of Dicloxacillin in human plasma at different therapeutic dose levels. The mean recovery of Dicloxacillin was found to be 98.98% ± 2.32. 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 Dicloxacillin to monitor drug concentration in body fluid, determination of drug level in plasma for dose regulation and bioavailability.

Acknowledgements:

Authors are thankful Maxim Pharmaceuticals, Pune of Dicloxacillin and acetanilide respectively. Authors are also thankful to Akshay Blood Bank, Pune for providing pooled human plasma and the Management, AISSMS College of Pharmacy, Pune, India for providing required facilities to carry out research work.

References:

1. Martindale –The complete drug reference, 35th Ed., Pharmaceutical Press, London (U.K), 2007, 265.
2. British Pharmacopoeia, Vol. I and II, HMSO Publication, London, (2007), pp265-266.
3. United States Pharmacopoeia NF (USP30, NF 25)1924.
4. Oscar Alderete, Dinora F. González-Esquivel, L. Misael Del Rivero, Nelly Castro Torres., Liquid chromatographic assay for dicloxacillin in plasma, Journal of Chromatography B, 2004, 805,353–356
5. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM) May 2001 BP.

*****