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Short communication

Liquid chromatographic assay for dicloxacillin in plasma

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Abstract

A simple high-performance liquid chromatographic method for the determination of dicloxacillin in plasma has been developed. The method only requires 0.5 ml of plasma, phosphate buffer solution (pH = 4.7), acidification with 0.5N hydrochloride acid and liquid extraction with dichloromethane. Posterior evaporation of organic under nitrogen steam and redissolution in mobile phase is carried out. The analysis was performed on a Spherisorb C_{18} (5 μm) column, using methanol–0.05 M phosphate buffer, pH = 4.7 (75:25; v/v) as mobile phase, with ultraviolet detection at 220 nm. Results showed that the assay is sensitive: 0.5 μg/ml. The response is linear in the range of 0.5–10 μg/ml. Maximum inter-day coefficient of variation was 12.4%. Mean extraction recovery obtained was 96.95%. Stability studies showed that the loss was not higher than 10%, samples are stable at room temperature for 6 h, at −20 °C for 2 months, processed samples were stable at least for 24 h and also after two freeze–thaw cycles. The method has been used to perform pharmacokinetic and bioequivalence studies in humans.

Keyword: Dicloxacillin

1. Introduction

The increased use of antibiotics has prompted the need for rapid, sensitive and specific methods for determining low concentrations in biological fluids. The available methods include microbiological assays, enzyme immunoassays, which require relatively long time for analysis or they do not have the required sensitivity [1,2] and high-performance liquid chromatography (HPLC) assays. The available HPLC methods for dicloxacillin reported include methods which are limited by complex requirements for sample preparation or by the use of chromatographic modifiers as well as methods for non-biological applications such as quality control [3–6]. Dicloxacillin (Fig. 1) is a high polar compound and their extraction is very difficult and problematic, also in order to obtain fast information about its pharmacokinetics it was developed a sensitive, specific, rapid and easy analytical method based on reversed-phase liquid chromatography and ultraviolet detection for the quantitation of dicloxacillin in plasma. The method was validated according to procedures and acceptance criteria based on FDA guidelines and recommendations of ICH [7,8].

The method has been used in bioequivalence studies with generic drugs and in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and solutions

Dicloxacillin salt (Lot 43B2) was obtained from COSUFAR (México, D.F., México) and flucloxacillin (internal estándar, I.S.) (Fig. 1) was kindly donated by Laboratorios Merck Sharp & Dohme (México). The purity of these standards compounds was higher than 99%. Acetonitrile for the mobile phase was of chromatographic grade (Mallinckrodt, Xalostoc México). All other reagents were analytical grade (Mallinckrodt, Xalostoc México). The following aqueous solutions were prepared: 0.05 M potassium phosphate buffer, pH adjusted to 4.7 with 0.8 M sodium hydroxide and 0.5N hydrochloride acid. Frozen drug-free, plasma for calibration curves was obtained from the hospital blood bank and thawed at room temperature before use.

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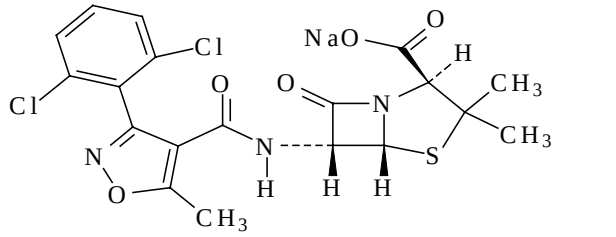
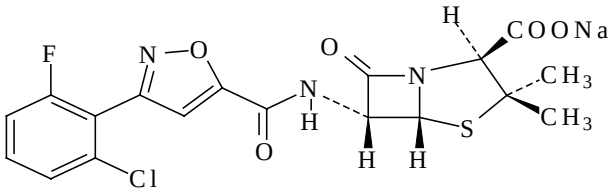


(A)



(B)

Fig. 1. Molecular structure of dicloxacillin (A) and flucloxacillin (B).

2.2. Chromatographic conditions

The instrument used was a Waters high-performance liquid chromatograph (Waters Associates, Milford, MA, USA), equipped with a variable wavelength UV-Vis detector (Breeze, model 2487), a binary delivery pump (Breeze model 1525), an autosampler-injector Plus (model 787) and a Chemstation Breeze v 3.2. Analysis was performed on a Spherisorb ODS-2 C_{18} column, 250 mm × 4.6 mm i.d.; 5 μm particle size (Waters Associates, Milford, MA, USA) with 0.05 M, pH = 4.7 phosphate buffer:acetonitrile (75:25; v/v) as the mobile phase. The column was kept at room temperature (20–25 °C) and the flow-rate was kept constant at 1.5 ml/min. The detection was recorded at 220 nm.

2.3. Sample preparation

To 0.1 ml of plasma spiked with dicloxacillin in the range of 0.5–10 μg/ml, 10 μl of methanolic solution of the internal standard, flucoxaxillin (200 μg/ml) were added, then 100 μl of 0.5 M phosphate buffer (pH = 4.7) were added and the sample was mixed, then 10 μl of 0.5N hydrochloride acid were added and the sample was mixed again. The sample was extracted with dichloromethane (1 ml) by mixing for 60 s. After centrifugation for 10 min at 1650 × *g*, the organic layer was separated and evaporated to dryness in a water bath at 40 °C under nitrogen and the residues were redissolved in 100 μl of mobile phase. Aliquots of 50 μl were injected into the HPLC system.

2.4. Method validation

2.4.1. Calibration curves

Stock solutions of analyte standards were prepared in methanol; working plasma solutions in the range from 0.5 to 10 μg/ml were prepared by adding different volumes from the stock dicloxacillin solution to blank plasma. The plasma sample was subject to the sample preparation procedure and injected into the HPLC. The procedure was carried out in triplicate for each concentration. Peak heights were recorded and the analyte/I.S. peak ratios obtained were plotted against the corresponding concentration of the analyte and the calibration curves were constructed by linear regression analysis.

The limit of quantitation (LOQ) and the limit of detection (LOD) were determined from the peak signal and the noise level, *S/N*, as10 and three times the baseline noise, respectively.

2.4.2. Extraction recovery

The procedure was the same as that described in Section 2.4.1. The analyte/I.S. peak ratio from plasma samples spiked with known amounts (0.5, 1.0, 2.0, 4.0, 5.0, 6.0, 9.0, 10.0 μg/ml) of the analyte were compared to the ratio obtained from standard solutions at the same theoretical concentration, injected directly onto the analytical column and the percent recovery calculated. Each sample was determined in triplicate.

2.4.3. Accuracy and precision

The intra-assay and inter-day precision and accuracy were evaluated by analyzing blank plasma spiked with different amounts of dicloxacilln and by calculating their concentration from a standard curve prepared on the same day. The inter-day assay was evaluated five times at three different concentrations, 1.0, 5.0 and 9.0 μg/ml on three different days.

2.4.4. Stability studies

The stability of dicloxacillin in plasma was evaluated with four studies: a short-term stability study, a long-term stability study, a freeze–thaw study and stability in the processed sample. Plasma blank samples were spiked with dicloxacillin at concentrations of 1.0, 5.0 and 9.0 μg/ml. Plasma samples extraction and subsequent HPLC analysis were carried out as described previously. They were determined by triplicate.

A short-term stability test was performed at room temperature, plasma samples spiked with dicloxacillin were at ambient temperature during 6 h and then extracted and analyzed. Their concentration was calculated from a standard curve produced on the same day.

The long-term stability study was carried out with plasma blank samples spiked with the drug, which were stored at −20 °C and they were analyzed periodically over span of 2 months against a standard curve prepared on the analysis day.

The freeze–thaw stability study was determined with spiked samples which were analyzed immediately after preparation, and after repeated freezing–thawing cycles at −20 °C.

The stability in the processed sample, ready for injection was determined at three levels of concentration, 1.0, 5.0, and 9.0 μg/ml at 12 and 24 h in triplicate.

3. Results and discussion

3.1. Chromatography and linearity

Chromatograms of plasma blank and plasma spiked with flucloxacillin, and dicloxacillin are shown in Fig. 2A and B. Retention times for the internal standard flucloxacillin, and dicloxacillin were 4.84 and 6.58 min, respectively. No interfering peaks at these times were detected. A linear relationship ($r = 0.9994$) was found when the ratio of the peak-height of dicloxacillin to the peak-height of the internal standard were plotted against various concentrations ranging from 0.5 to 10 μg/ml.

3.2. Precision and recovery

Intra-day and inter-day (3 days) precision and accuracy of the method, was assessed by analizing quality control samples spiked with known amounts of dicloxacillin. Results are shown in Tables 1 and 2, it can be seen that the maximum intra-day coefficient of variation was 5.87% at 0.5 μg/ml. In the same way, the maximum inter-day coefficient of variation was 12.4% at 1.0 μg/ml.

The mean recovery calculated at eight different concentrations (0.5–10 μg/ml) was 96.95% ($n = 3$).

Table 1
Precision and recovery of the HPLC method in plasma samples

Theoretical concentration (μg/ml)	Experimental concentration mean (μg/ml)	C.V. (%)	Recovery (%)	Absolute deviation (%)
Intra-day ($n = 3$)				
0.5	0.45	5.87	90.0	10
1.0	0.91	5.11	91.38	8.6
2.0	1.94	1.94	97.16	2.8
4.0	4.12	4.14	103.0	3.0
5.0	4.81	6.99	96.34	3.6
6.0	6.04	2.15	100.66	0.66
9.0	8.67	2.7	96.42	3.57
10.0	9.94	0.56	99.4	0.6

Table 2
Accuracy and inter-day precision of the analytical method for dicloxacillin ($n = 3$)

Concentration added (μg/ml)	Accuracy (%)	Inter-day recovery (%)	C.V. inter-day (%)
1	96.36	105.4	12.4
5	97.28	103.2	6.2
9	98.7	99.75	4.0

C.V.: coefficient of variation.

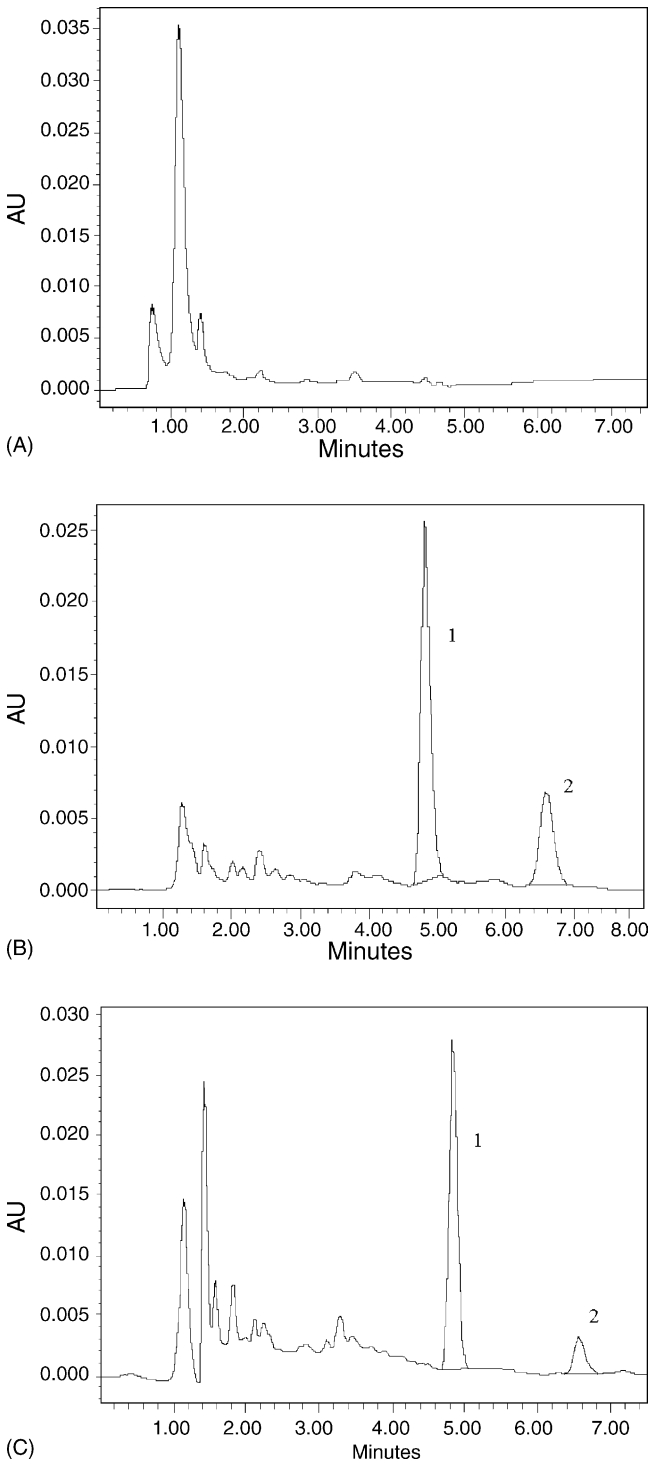


Fig. 2. Chromatograms of (A) blank plasma, (B) plasma spiked with dicloxacillin [1] and flucloxacillin [2] as I.S., and (C) plasma sample from a human volunteer receiving 500 mg of dicloxacillin.

3.3. Limit of detection and limit of quantitation

The limit of detection defined as three times the baseline noise was 0.2 μg/ml. The limit of quantitation was 0.5 μg/ml ($n = 3$, C.V. less than 15%).

3.4. Stability studies

In order to determine the stability of dicloxacillin in plasma four studies were carried out: a short-term stability study, a long-term stability study, a freeze–thaw study and stability in the processed sample. For the stability study plasma samples were spiked with dicloxacillin at concentrations of 1.0, 5.0 and 9.0 μg/ml and they were determined in triplicate.

A short-term stability test performed at room temperature showed that the samples spiked with dicloxacillin at concentrations of 1.0, 5.0 and 9.0 μg/ml were stable for 6 h (average recoveries were: 90.25, 100.23 and 94.03%, respectively).

The long-term stability of the dicloxacillin in plasma from samples stored at −20 °C was determined by periodic analysis over span of 2 months. Samples were analyzed immediately after preparation and after storage. The results indicated that dicloxacillin samples were stable for 2 months, with an average recovery of 94.31%.

The freeze–thaw stability was also determined, spiked samples were analyzed immediately after preparation, and on a daily basis, after repeated freezing–thawing cycles at −20 °C on 2 consecutive days. At least two freeze–thaw cycles can be tolerated without a loss higher than 10%. The total average recovery was 92.22%.

Finally, the stability in the processed sample ready for injection, was determined at three levels of concentration, 1.0, 5.0, and 9.0 μg/ml at 12 and 24 h in triplicate. Result showed that these samples are stable at least during 24 h. The loss was not higher than 10%.

The method was used in the plasma analysis of a pharmacokinetic study of dicloxacillin. Fig. 2C shows a typical chromatogram obtained in humans after the oral administration of a single dose of 500 mg of dicloxacillin and Fig. 3 shows the mean plasma levels of dicloxacillin in healthy volunteers. Maximum plasma concentration in plasma ranged from 4.9 to 6.3 μg/ml at 0.75–1 h. The half-life ranged from 0.7 h to 0.92 h. Also the mean value of area under the concentration–time curve (AUC) obtained was 13.728 ± 5.61 μg/ml. There was no chromatographic interference from any endogenous compound.

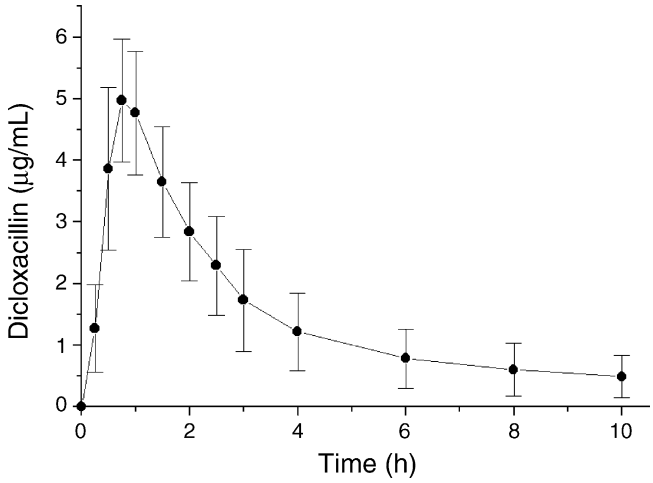


Fig. 3. Mean plasma concentration–time curve following oral administration of dicloxacillin (500 mg) in 24 healthy volunteers.

4. Conclusions

The method is simple, rapid and sensitive. The principal advantage of the method is the use of a simple liquid–liquid sample preparation, which is very easy and fast. It can be also used as a reliable assay in the study of pharmacokinetics of dicloxacillin as well as in bioavailability/bioequivalence studies.

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