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RP-HPLC analysis of flucloxacillin in human plasma: validation and application to a bioequivalence study

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A RP-HPLC method with rapid sample processing was developed for quantitation of flucloxacillin in human plasma using dicloxacillin as the internal standard. The plasma sample (100 μL) was acidified with glacial acetic acid, and deproteinized by precipitation with acetonitrile. The supernatant was directly injected into the HPLC system. Separation was achieved on an Alltima™ C_{18} column (250 mm × 4.6 mm I.D., 5 μm), with a mixture of 10 mmol · L^{-1} KH_2PO_4-acetonitrile (64.5 : 35.5, v/v) as mobile phase. The assay was successfully applied to a randomized, two-period cross-over bioequivalence study in 20 healthy Chinese volunteers following a single oral dose of 250 mg flucloxacillin capsules. A non-compartmental method was used for pharmacokinetic analysis. Compared with data in the literature, flucloxacillin was eliminated more slowly in Chinese than in Caucasians. C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ were tested for bioequivalence after log-transformation of data. No significant difference was found. T_{max} was analyzed by Wilcoxon's test and no significant difference was obtained ($P > 0.05$). Based on these statistical inferences, the two formulations were judged to be bioequivalent and, thus, can be prescribed interchangeably.

1. Introduction

Flucloxacillin is a beta-lactamase stable isoxazolyl-penicillin. It is indicated for the treatment of infections due to staphylococci resistant to benzylpenicillin and mixed streptococcal infections (Bodey et al. 1972; Bal and Gould 2005). It is acid-stable and can be administered orally. Its assay methods in the literature include microbiological assays (Roder et al. 1995; Frank et al. 1988) and HPLC involving liquid-liquid or solid-phase extraction (Charles et al. 1994; Hung et al. 1988). These methods are limited by complex requirements for sample preparation and a relatively long time for analysis. Furthermore, bioequivalence studies of oral flucloxacillin have not been reported in the literature and its pharmacokinetics in Chinese have not yet been described.

The objective of the present investigation is to establish a fully validated HPLC method with a sufficiently low quantitation limit and shorter time for sample preparation to support pharmacokinetic and bioequivalence studies of flucloxacillin.

2. Investigations and results

2.1. Assay validation

Fig. 1 shows representative chromatograms of blank plasma, a plasma sample spiked with flucloxacillin (5 mg · L^{-1}) and a plasma sample obtained at 0.5 h after a single 250 mg oral dose of flucloxacillin in a healthy volunteer. Typical retention times for flucloxacillin and the IS were 6.5 and 7.9 min, respectively. The total run time was 10 min. The peak shapes were sharp and symmetrical. Flucloxacillin and IS were well separated from endogenous compounds under the chromatographic conditions described. Compared with its parent drug, 5-hydroxymethylflucloxacillin, the major metabolite of flucloxacillin, has less lipid solubility and hence a shorter retention time. Under the HPLC conditions specified, it did not interfere with flucloxacillin.

The assay was specific and linear from 0.2 to 40.0 mg · L^{-1}. The mean linear regression equation of the calibration curve for flucloxacillin was $Y = 0.1081\ (\pm 0.0094)\ X - 0.00219\ (\pm 0.00281)$ ($n = 5$, $r = 0.9999$), where Y is the peak area ratio of flucloxacillin to the IS and X is the flucloxacillin concentration. LLOQ was established as 0.2 mg · L^{-1}.

Precision and accuracy of flucloxacillin in quality control (QC) samples (Table 1) fell well within the limits of acceptability. All values were <11%. The mean absolute recovery was 87.2% for flucloxacillin.

The stock solutions of flucloxacillin and IS were stable for at least 60 d when stored at −20 °C. Stability results for flucloxacillin in plasma are shown in Table 2, indicating that flucloxacillin was stable in plasma samples under different storage conditions: immediately, after 3 h at ambient temperature, after sample processing and being on the autosampler at 5 °C for 18 h, after three freeze/thaw cycles and after 1 mo stored at −20 °C.

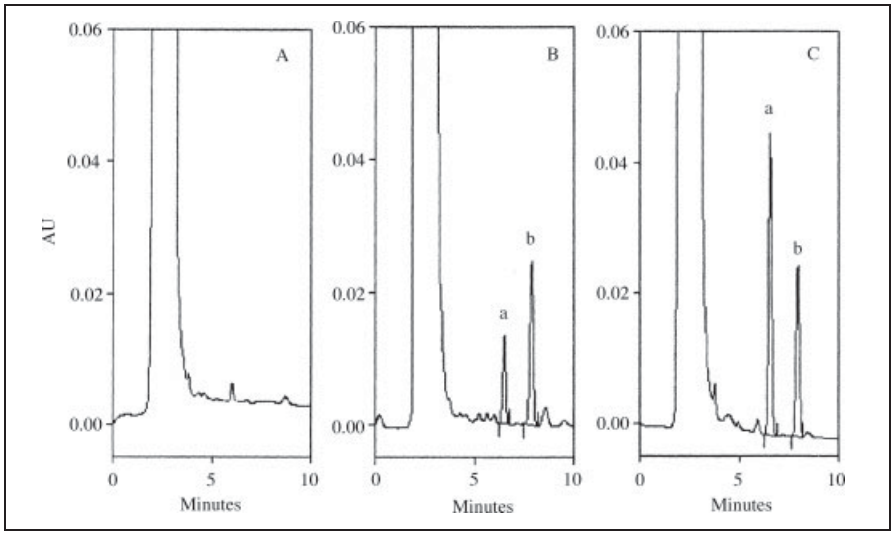


Fig. 1:
Chromatograms
A-blank plasma; B-5 mg · L^{-1} standard sample of flucloxacillin; C-plasma sample at 0.5 h after a single oral dose of flucloxacillin capsule (250 mg) for one volunteer
a flucloxacillin, b internal standard

Table 1: Precision and accuracy of flucloxacillin in quality control samples

Added conc. (mg · L^{-1})	Between-batch conc. measured (mg · L^{-1}, n = 18)	RSD (%)	RE (%)	Within-batch conc. measured (mg · L^{-1}, n = 6)	RSD (%)	RE (%)
0.4	0.42	10.5	5.0	0.39	10.4	−2.5
4.0	4.01	6.0	0.3	3.94	6.8	−1.5
30.0	30.2	5.1	0.7	28.5	3.8	−5.0

Table 2: Stability of flucloxacillin in human plasma (n = 3)

Stability study	Added conc. (mg · L^{-1})	Found conc. (mg · L^{-1})	RSD (%)	RE (%)
Short-term	0.4	0.42	10.4	−4.6
stability for 3 h in	4.0	3.92	2.1	1.5
plasma	30.0	31.64	1.3	4.2
Three freeze-thaw	0.4	0.42	10.0	4.6
cycles	4.0	3.88	2.6	−2.8
	30.0	29.36	0.7	−4.4
Autosampler	0.4	0.41	7.6	−6.8
stability for 18 h	4.0	3.81	8.0	−5.3
	30.0	28.54	2.6	−4.9
30 days' stability	0.4	0.39	8.0	−2.5
at −20 °C	4.0	3.88	1.8	−3.7
	30.0	30.46	1.6	0.3

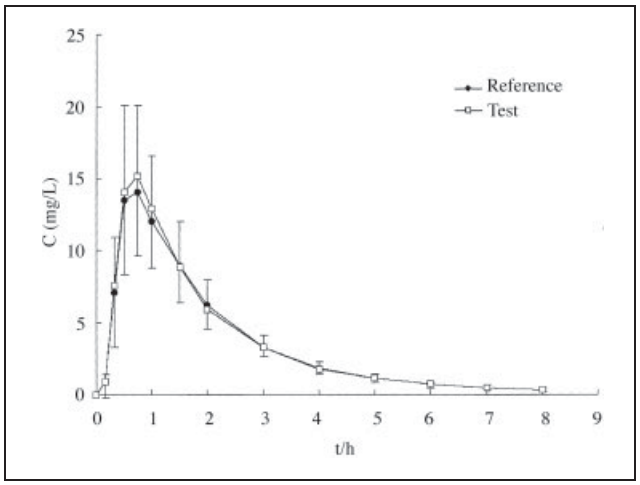


Fig. 2: Mean concentration-time curve of flucloxacillin after a single oral dose of 250 mg flucloxacillin capsules in 20 healthy volunteers

2.2. Bioequivalence

The average concentration-time curves are shown in Fig. 2. Major pharmacokinetic parameters derived from the two formulations are listed in Table 3. Flucloxacillin had a rapid absorption profile in Chinese volunteers. The residual area (differences between $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$) of flucloxacillin (reference: 3%; test: 4%) accounted for <20% of the $AUC_{(0-t)}$. Therefore, the stated criterion ($AUC_{(0-t)}/AUC_{(0-\infty)} \cdot 100\% > 80\%$) was fulfilled and the residual area had no significant impact on the calculation of $AUC_{(0-\infty)}$ and, consequently, on bioavailability.

Table 3: Pharmacokinetic parameters obtained from 20 Chinese volunteers after administration of 250 mg flucloxacillin sodium capsules

Parameters	Test	Reference
$AUC_{(0-t)}$ (mg · h · L^{-1})		
Geom. mean	28.7	27.9
S.D.	1.3	1.4
$AUC_{(0-\infty)}$ (mg · h · L^{-1})		
Geom. mean	29.8	28.8
S.D.	1.3	1.3
$AUC_{(0-t)}/AUC_{(0-\infty)}$ (%)		
Mean	96.3	96.8
S.D.	2.5	2.8
$AUC_{(t-\infty)}/AUC_{(0-\infty)}$ (%)		
Mean	4	3
S.D.	2	3
C_{max} (mg · L^{-1})		
Geom. mean	14.5	13.9
S.D.	1.5	1.6
$T_{1/2}$ (h)		
Mean	1.9	1.7
S.D.	0.6	0.4
T_{max} (h)		
Mean	0.7	0.8
S.D.	0.1	0.4

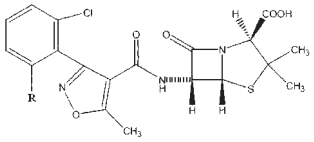
Table 4: ANOVA for assessment of the product, subject and period effects, point estimate and 90% CI for the test/reference ratio of C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$, using logarithmic transformed data, after administration of 250 mg flucloxacillin sodium capsules to 20 Chinese volunteers ($\alpha = 0.05$)

Parameters	ANOVA (P-value) Variation source			Point estimate	90% CI	Power
	Product	Subject	Period			
C_{max}	0.7084	0.0135	0.1736	105.5%	87.1%–124.1%	0.8150
$AUC_{(0-t)}$	0.6483	0.0058	0.2715	101.4%	92.6%–114.2%	0.9748
$AUC_{(0-\infty)}$	0.5610	0.0046	0.2867	101.0%	93.8%–114.1%	0.9717

Table 4 lists data for assessment of product, subject and period effects; point estimate as well as 90% CIs of the ratios (test/reference) for the log-transformed values; and the power of the test. No statistically significant formulation or period effect was encountered ($P > 0.05$). Inter-subject differences were the main source of variability in log-transformed C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$. The 90% CI for the ratio of C_{max} (87.1%–124.0%), $AUC_{(0-t)}$ (92.6%–114.2%), and $AUC_{(0-\infty)}$ (93.8%–114.1%) values for the test and reference products were also entirely within the FDA acceptable range of 80%–125%. In addition, no significant difference was obtained for T_{max} ($P > 0.05$).

3. Discussion

The chemical structure of dicloxacillin is very similar to that of flucloxacillin, so we tried using dicloxacillin as the IS. The working solution of IS was found to be unstable and its content decreased by 30% after storage at 4 °C for 48 h. However, it was stable for at least 48 h if stored at −20 °C.



Flucloxacillin R= F
Dicloxacillin R= Cl

Flucloxacillin and IS are acid-stable semisynthetic penicillins. Plasma protein binding of both flucloxacillin and IS was high (94.7 ∼ 97.2%) (Roder et al. 1995). It was presumed that acidification could facilitate freeing these two weakly acidic drugs from plasma albumin. Thus, we tried sample preparation based on acidifying plasma and direct protein precipitation. Both analyte and IS had a very weak peak signal if the sample was processed without acidification, whereas their peak signal improved markedly if the sample was processed with glacial acetic acid (Fig. 3). It showed that acidification improved the absolute recovery of both the analyte and the IS in the plasma sample.

Sufficient recovery could be achieved using the sample preparation method established, whereas increasing the volume of protein-precipitator (i.e., acetonitrile) brought about not higher recovery but lower assay sensitivity. Column pressure was basically stable during the analysis of 800 plasma samples.

The IS was found to be more sensitive to acetonitrile content was than flucloxacillin and a mixture of 10 mmol · L^{-1} KH_2PO_4-acetonitrile (64.5 : 35.5, v/v) achieved good resolution and symmetrical peak shapes of the analytes as well as a short run time.

Flucloxacillin has strong antimicrobial activities against *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. MIC_{90} values have been quoted as 0.1 ∼ 0.5 mg · L^{-1} (Sutherland et al. 1970; Steer et al. 1997). In our study, the mean plasma concentrations of flucloxacillin in the elimination phase (i.e., 5, 6, 7 and 8 h after oral dose) were 1.2, 0.7, 0.5 and 0.35 mg · L^{-1}, respectively. It is well known that the clinical efficacy of β-lactams is highly related to the amount of time for which the serum concentration exceeds the MIC_{90} ($T > MIC_{90}$) (Turnidge 1998; Nicolau 2001). The results of our study provided evidence for the prescribing information that oral 250 mg flucloxacillin should be administered four times daily. A pronounced inter-subject variability in C_{max} was observed, with the values ranging from 3.2 ∼ 26.0 mg · L^{-1}. However, plasma concentration of flucloxacillin at 6 h after oral dose in each subject was well above 0.5 mg · L^{-1}. Thus, clinical efficacy was still warranted in treatment of infections caused by susceptible bacteria.

Mean T_{max} and C_{max} in Chinese volunteers were 0.7 ∼ 0.8 h and 15 mg · L^{-1}, respectively, very similar to the pharmacokinetic properties described for 250 mg flucloxacillin capsules (Athlone Laboratories, Ireland). Mean $T_{1/2}$ (1.7 ∼ 1.9 h) in this study was longer than the half-life in Caucasians, e.g., 1.31 ∼ 1.39 h (Paton et al. 1982), 0.75 ∼ 1 h specified in the 50th edition of the British National Formulary and 0.75 ∼ 0.83 h described for flucloxacillin capsules (Athlone Laboratories, Ireland). It indicated that flucloxacillin is eliminated more slowly in Chinese than in Caucasians.

Two drug products are considered to be bioequivalent and thus therapeutically equivalent if they are pharmaceutically equivalent and if their rates (C_{max}) and extents of absorption (AUC) do not show a significant difference when administered at the same molar dose of the therapeutic moiety under similar experimental conditions (Chow and Liu 1992). Based on the above mentioned statistical inferences, the two formulations were judged to be bioequivalent and, thus, can be prescribed interchangeably.

In summary, an RP-HPLC method with rapid sample processing to determine flucloxacillin levels in human plasma

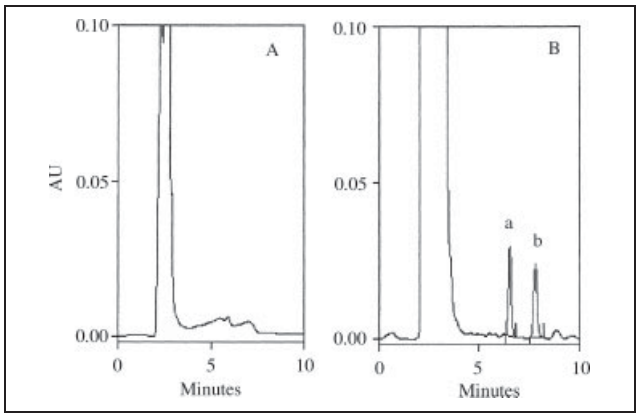


Fig. 3: Chromatograms of 10 mg · L^{-1} standard sample of flucloxacillin A- without adding glacial acetic acid; B- adding glacial acetic acid a- flucloxacillin; b- internal standard

was established, validated and successfully applied to a bioequivalence study. The assay developed showed acceptable precision, accuracy, linearity, stability and specificity. A pharmacokinetic and bioequivalence study in Chinese is firstly reported for the first time.

4. Experimental

4.1. Drugs and reagents

Flucloxacillin (91.6% purity) and dicloxacillin (90.6% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dicloxacillin was used as the IS. HPLC-grade acetonitrile and methanol were purchased from Caledon (Georgetown, Canada). Ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade. Test formulation was flucloxacillin sodium capsules (lot 060110) from Shandong Pharmaceutical Factory (Shandong, China). Reference formulation was flucloxacillin sodium capsules (lot 050737) from Bright Future Pharmaceutical Laboratories Ltd. (Hong Kong, China). Each capsule contained 250 mg of flucloxacillin sodium.

4.2. Standard solutions

A standard stock solution of flucloxacillin was prepared by dissolving 10.9 mg flucloxacillin in 10 mL methanol to give a nominal concentration of 1 $mg \cdot mL^{-1}$ and the solution was kept at 4 °C before use. The IS stock solution was prepared by dissolving 11.0 mg dicloxacillin in 10 mL methanol to give a nominal concentration of 1 $mg \cdot mL^{-1}$. The IS working solution (20 $mg \cdot L^{-1}$) was prepared by diluting the stock solution of IS with acetonitrile. Both IS stock solution and IS working solution were kept at −20 °C before use.

4.3. Chromatography

The HPLC system consisted of a System Gold 125 solvent module, a model 508 autosampler, a 166 UV detector and 32 Karat™ Software version 5.0 (Beckman Coulter, Inc., Fullerton, USA) and an AT-330 column oven (Autoscience Co., Tianjin, China). The separation of compounds was made on an Alltima® (Alltech Associates, Inc., USA) ODS C_{18} column (5 μm, 250 mm × 4.6 mm i.d.), protected by a security guard cartridge ODS C_{18} 4 mm × 3.0 mm ID (Phenomenex. Inc., USA). The mobile phase was a mixture of 10 $mmol \cdot L^{-1}$ KH_2O_4-acetonitrile (64.5 : 35.5, v/v) pumped at a flow-rate of 1.0 $mL \cdot min^{-1}$. The column oven was kept at 35 °C. The autosampler was set at 5 °C. The UV detector was set at 220 nm.

4.4. Sample preparation

A 100 μL volume of plasma was transferred to a microcentrifuge tube (Eppendorf, 1.0 mL), and then 50 μL of IS working solution was spiked and vortex-mixed for 5 s. A 50 μL aliquot of glacial acetic acid and 250 μL of acetonitrile were sequentially added. The sample was vortex-mixed again for 10 s. After centrifugation at 36670 g for 10 min at 15 °C, the supernatant was transferred to vials on the rack of the autosampler and a 50 μL aliquot was injected into the chromatographic system.

4.5. Assay validation

Standard plasma samples of flucloxacillin at concentrations of 0.2, 0.5, 1, 5, 10, 20 and 40 $mg \cdot L^{-1}$ were obtained by diluting the stock solution of flucloxacillin with drug-free human plasma. The QC samples were prepared in bulk with an independent weighing of standard drug at concentrations of 0.4 (low), 4 (medium) and 30 $mg \cdot L^{-1}$ (high) for flucloxacillin. The calibration and QC samples were divided into aliquots in microcentrifuge tubes and stored in the freezer at below −20 °C until analysis.

A calibration curve was constructed from a blank sample (a plasma sample processed without an IS) and seven standard plasma samples covering the total range (0.2 − 40 $mg \cdot L^{-1}$ for flucloxacillin). Peak areas were recorded and the analyte/IS peak area ratios obtained were plotted against the corresponding concentration of the analyte. The calibration curves were constructed by linear regression analysis on five consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient of 0.99 or better, and that each back-calculated standard concentration must be within a 15% deviation from the nominal value except for the LLOQ, for which the maximum acceptable deviation was set at 20%. LLOQ was determined from the peak signal and the noise level, S/N, as 10.

Six randomly selected blank human plasma samples were carried through the assay procedure to determine the extent to which endogenous plasma components may contribute to interference with the analyte or IS. The results were compared with the LLOQ for flucloxacillin.

Absolute recovery of flucloxacillin was evaluated by comparing the analyte to IS peak area ratios of six processed low-, medium- and high-QC samples to the ratios obtained from standard solutions fortified with glacial acetic acid at the same theoretical concentrations of flucloxacillin.

Within-batch accuracy and precision evaluations were performed by repeated analysis of flucloxacillin in human plasma. The run consisted of a calibration curve plus six replicates of each of the low-, medium- and high-QC samples. Between-batch accuracy and precision were assessed by analysis of samples consisting of a calibration curve and six replicates of low-, medium- and high-QC samples for flucloxacillin on three separate occasions.

The stability of flucloxacillin in plasma was evaluated in four studies: a short-term stability study, a long-term stability study, a freeze-thaw study and stability in the processed sample. The low-, medium- and high-QC samples for flucloxacillin were assayed in triplicate. The concentration of flucloxacillin after each storage period was related to the initial concentration as determined for samples that were freshly prepared and processed immediately. The stability of standard stock solutions was also tested after storage at −20 °C and under refrigeration (4 °C).

4.6. Bioequivalence study

After approval by the Ethics Committee of the 2nd Affiliated Hospital (School of Medicine, Zhejiang University), 20 healthy male Chinese volunteers gave written informed consent to participate in a randomized, two-period cross-over bioequivalence study. After a 12-hour overnight fast, the volunteers received a single oral dose (equivalent to 250 mg of flucloxacillin) of the test or reference products with 200 mL water. No food was allowed until 4 h after dose administration. Blood samples (1 mL) were drawn into Vacutainer™ tubes containing K_2EDTA from a forearm vein using an indwelling catheter before drug intake and at 0.17, 0.33, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 h after dosing. Blood samples were centrifuged at 3000 g for 10 min and plasma was separated and stored at −80 °C until HPLC determination.

Pharmacokinetic parameters were calculated using of a non-compartmental method. Bioequivalence evaluation was performed using BAPP2.0 software (Center of Drug Metabolism & Pharmacokinetics, China Pharmaceutical University, Nanjing, China). C_{max} and T_{max} were determined by inspection of the plasma concentration-time profile. The terminal elimination rate constant (λ_z) was determined by linear regression of the terminal portion of the log concentration-time profile. $T_{1/2}$ was calculated as $0.693/\lambda_z$. AUC was determined by the trapezoidal rule and extrapolated to infinity by calculation of C_t/λ_z. Log transformed C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ were tested for bioequivalence using ANOVA and Schuirmann's two-one sided t-test (Schuirmann 1987). T_{max} was analyzed by Wilcoxon's test. A P value of less than 0.05 was considered to be statistically significant. If the two formulations were bioequivalent, 90% CI values for test/reference ratios of C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ should fall within the range of 80% − 125% (Chow and Liu 1992; Food and Drug Administration 1993).

References

Bal AM, Gould IM (2005) Antibiotic resistance in *Staphylococcus aureus* and its relevance in therapy. Expert Opin Pharmacother 6: 2257–2269.

Bodey GP, Vallejos C, Stewart D (1972) Flucloxacillin: a new semisynthetic isoxazolyl penicillin. Clin Pharmacol Ther 13: 512–515.

Charles BG, Foo CC, Gath J (1994) Rapid column liquid chromatographic analysis of flucloxacillin in plasma on a microparticulate pre-column. J Chromatogr B Biomed Appl 660: 186–190.

Chow CS, Liu JP (2000) Statistical methods for average bioavailability. In: Chow CS, Liu JP (ed.) Design and Analysis of Bioavailability and Bioequivalence Studies, 2nd ed., revised and expanded. New York, p. 79–124.

Food and Drug Administration (1993) In vivo bioequivalence guidances. Pharmacopeial Forum 19: 6501–6508.

Frank U, Schmidt-Eisenlohr E, Schlosser V, Spillner G, Schindler M, Daschner FD (1988) Concentrations of flucloxacillin in heart valves and subcutaneous and muscle tissues of patients undergoing open-heart surgery. Antimicrob Agents Chemother 32: 930–931.

Hung CT, Lim JK, Zoest AR, Lam FC (1988) Optimization of high-performance liquid chromatographic analysis for isoxazolyl penicillins using factorial design. J Chromatogr 425: 331–341.

Nicolau DP (2001) Predicting antibacterial response from pharmacodynamic and pharmacokinetic profiles. Infection 29 (Suppl 2): 11–15.

Paton DM, Mander P, Avery RJ (1982) Bioavailability and half-life of two preparations of flucloxacillin. N Z Med J 95: 766–768.

Roder BL, Frimodt-Moller N, Espersen F, Rasmussen SN (1995) Dicloxacillin and flucloxacillin: pharmacokinetics, protein binding and serum bactericidal titers in healthy subjects after oral administration. Infection 23: 107–112.

Schuirmann DJ (1987) A comparison of the two one-sided tests procedure and the power approach for assessing the equivalence of average bioavailability. J Pharmacokinet Biopharm 15: 657–680.

Steer JA, Papini RP, Wilson AP, McGrouther DA, Parkhouse N (1997) Teicoplanin versus flucloxacillin in the treatment of infection following burns. J Antimicrob Chemother 39: 383–392.

Sutherland R, Croydon EAP, Rolinson GN (1970) Flucloxacillin, a new isoxazolyl penicillin, comparaed with oxacillin, cloxacillin, and dicloxacillin. Br Med J 4: 455–460.

Turnidge JD (1998) The pharmacodynamics of beta-lactams. Clin Infect Dis 27: 10–22.