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OPTIMIZATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS FOR ISOXAZOLYL PENICILLINS USING FACTORIAL DESIGN

C.T. HUNG*, J.K.C. LIM and A.R. ZOEST

Department of Pharmacy, University of Otago, Dunedin (New Zealand)

and

F.C. LAM

Department of Mathematics and Statistics, University of Otago, Dunedin (New Zealand)

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SUMMARY

A 3×3 factorial design has been used to study the effects of pH and acetonitrile concentration of the eluents on the retention and resolution of cloxacillin, flucloxacillin and dicloxacillin on a C_{18} column. The logarithm of the capacity factors of these solutes have been found to vary linearly with the pH and quadratically with the acetonitrile content. The equations generated have been employed to predict experimental conditions necessary for an optimum separation. The chromatographic condition selected has been applied to the quantitation of flucloxacillin in human plasma using dicloxacillin as the interval standard. Sample preparation consists of protein precipitation and solid-phase extraction. The detection limit of the assay at 220 nm for flucloxacillin is in the region of 0.1 μg/ml. This assay has been employed in a study of the relative bioavailability of two commercial flucloxacillin sodium capsules in ten healthy volunteers.

INTRODUCTION

The widespread use of benzylpenicillin in the past forty years has led to the emergence of resistent bacterial strains. Isoxazolyl penicillins are now commonly used for the treatment of penicillin-resistant *Staphylococcus aureus* infections. In spite of their clinical application for more than twenty years, there are only a few reported high-performance liquid chromatographic (HPLC) assays for these compounds in human plasma [1,2]. In addition, all reported liquid chromatographic analysis for isoxazolyl penicillins were developed empirically and the experimental conditions employed might not have been at their optima.

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In this study, a factorial approach [3] has been used for the optimization of the retention and separation of cloxacillin, flucloxacillin and dicloxacillin by reversed-phase HPLC. Information obtained from the optimization study has also been applied to the plasma sample pretreatment using bonded silicas. The assay developed has been employed in a study comparing the bioavailability of two commercial flucloxacillin sodium capsules.

EXPERIMENTAL

Apparatus and materials

The chromatographic system consisted of an LKB 2150 HPLC pump (Stockholm, Sweden) and a Shimadzu (Kyoto, Japan) Model SPD-6A variable wavelength detector. Samples were introduced via a Waters 712 WISP autoinjector (Milford, MA, U.S.A.) or a Rheodyne 7125 injector (Cotati, CA, U.S.A.) with a 20-μl sample loop. The chromatographic column, 100 mm $\times$ 2 mm I.D., was slurry packed with 5-μm ODS-Hypersil (Shandon, Cheshire, U.K.) and had efficiency of over 4000 plates per 10 cm. Bond Elut® cartridges (C_{18}) used for plasma sample clean-up were obtained from Analytichem (Harbor City, CA, U.S.A.).

Cloxacillin sodium and flucloxacillin sodium were provided by Glaxo Pharmaceuticals (Palmerston North, New Zealand) Dicloxacillin sodium was purchased from Sigma (St. Louis, MO, U.S.A.). Orthophosphoric acid and disodium hydrogenphosphate were obtained from BDH (Poole, U.K.). Acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was double glass-distilled and Milli-Q®-filtered. All reagents were of Analar or equivalent grade. The generic flucloxacillin sodium capsules (PL 0039 0162) were supplied by Evans Medical (Palmerston North, New Zealand) and Floxapen® capsules (95931: XE 10) were from Beecham Research Labs. (Auckland, New Zealand). Both capsules contained 500 mg flucloxacillin as its sodium salt.

Factorial design

A 3 $\times$ 3 factorial design was employed in the optimization of the eluents with pH levels at 2, 4 and 6, and acetonitrile concentration at 20, 40 and 60% (v/v). In addition to the above nine solvents, two extra eluents at the centre point, i.e. at pH 4 and 40% (v/v) acetonitrile, were also included in the experiment to estimate the error involved in the preparation of the mobile phases. The disodium hydrogenphosphate buffer concentration in the eleven eluents was fixed at 10 m*M*. The eluents were adjusted to the appropriate pH with orthophosphoric acid. The experiments were conducted in a temperature-controlled room at 28 $\pm$ 2°C. The eleven eluents prepared were then randomized for testing. The retention time of the test compounds in each mobile phase was measured in triplicate and if an individual measurement differed from the other by 5%, further replicate measurements were performed. The data obtained were analysed using the SAS computer package [4].

Flucloxacillin bioavailability study

Ten healthy male volunteers aged between 20 and 25 years participated in a two-way cross-over bioavailability study. The subjects were divided into two groups. Group 1 received the 500-mg generic flucloxacillin sodium capsules first followed by 500 mg Floxapen. Group 2 received 500 mg Floxapen first, followed by the generic product. One week separated the administration of the two dosage forms. Blood samples (10 ml) were collected through a catheter from each subject at scheduled intervals over 10 h after drug administration. The heparinized blood samples were then centrifuged and the plasma samples collected and frozen at −15 °C until assayed. The frozen flucloxacillin plasma samples were found to be stable for at least two months.

Sample preparation

To 250 μl of plasma sample in a 1.5-ml plastic centrifuge tube, 100 μl of the internal standard solution (20 μg/ml dicloxacillin sodium in water) were added. The plasma was deproteinated using 400 μl of acetonitrile at −15 °C while vortexing. A 700-μl volume of 10 m*M* disodium hydrogenphosphate buffer (pH 2) was then added and the sample centrifuged for 10 min at 8000 *g*. The supernatant was further purified using 1-ml Bond Elut C_{18} column. The column was conditioned with 2 ml of acetonitrile and 1 ml of 10 m*M* disodium hydrogenphosphate buffer (pH 2) before the sample introduction. Following the sample introduction, the column was washed with 1 ml distilled water. The flucloxacillin and the internal standard, dicloxacillin, were then eluted with 2×500 μl of acetonitrile–water (35:65, v/v) containing 10 m*M* disodium hydrogenphosphate and adjusted to pH 6 with phosphoric acid. The eluent collected (25 μl) was then injected onto the column.

Chromatography

The processed plasma samples were analysed for flucloxacillin using a mobile phase of acetonitrile–water (40:60, v/v) containing 10 m*M* disodium hydrogenphosphate and adjusted to pH 2 with orthophosphoric acid. A flow-rate of 0.5 ml/min was employed and detection was at 220 nm.

RESULTS AND DISCUSSION

Factorial design

Of all the bonded stationary phases used by chromatographers, the C_{18} silica is by far the most popular [5]. ODS-Hypersil, being a typical modern capped octadecyl material, was thus selected as the stationary support. Since the experiments were conducted in a constant-temperature environment, the remaining important factors that govern the retention and separation of acidic compounds, such as isoxazolyl penicillins ($pK_a \sim 2.7$), in the reversed-phase HPLC system were the organic modifier concentration and the pH of the eluent [6].

The retention data of the three isoxazolyl penicillins, obtained under all the experimental conditions, are presented in Table I. The logarithm of the observed capacity factor ($\ln k'$) of each of the three drugs was fitted to a second-order

TABLE I

CAPACITY FACTORS (k') OF CLOXACILLIN, FLUCLOXACILLIN AND DICLOXACILLIN IN THE ELEVEN ACETONITRILE-BUFFER MOBILE PHASES, EACH CONTAINING 10 m*M* DISODIUM HYDROGEN PHOSPHATE

Chromatographic conditions: 100 mm × 2 mm ODS-Hypersil column; flow-rate, 0.5 ml/min; temperature, 28 ± 2 °C.

Mobile phase		k'		
pH	% Acetonitrile	Cloxacillin	Flucloxacillin	Dicloxacillin
2	20	127.22	167.89	302.78
2	40	4.15	5.07	6.93
2	60	0.89	0.94	1.19
4	20	23.67	31.89	56.33
4	40	1.78	2.22	3.11
4	40	1.61	1.91	2.51
4	40	1.50	1.80	2.41
4	60	0.61	0.72	0.78
6	20	8.89	12.44	21.44
6	40	0.33	0.39	0.47
6	60	0.22	0.22	0.28

polynomial with respect to the acetonitrile composition and the pH of the eluents. After deleting the statistically non-significant terms ($p < 0.05$), the following equations for the three compounds were obtained:

$$\ln k' \text{ (cloxacillin)} = 10.71 - 0.55\,\text{pH} - 0.31\ (\%\ \text{acetonitrile}) + 0.0026\ (\%\ \text{acetonitrile})^2 \quad (1)$$

$$\ln k' \text{ (flucloxacillin)} = 11.16 - 0.55\,\text{pH} - 0.31\ (\%\ \text{acetonitrile}) + 0.0026\ (\%\ \text{acetonitrile})^2 \quad (2)$$

$$\ln k' \text{ (dicloxacillin)} = 12.25 - 0.57\,\text{pH} - 0.34\ (\%\ \text{acetonitrile}) + 0.0028\ (\%\ \text{acetonitrile})^2 \quad (3)$$

The capacity factor maps for cloxacillin, flucloxacillin and dicloxacillin, as a function of pH and acetonitrile concentration of the eluent, are presented in Fig. 1. At the pH range studied, i.e. pH 2–6, the ln k' of the solutes has a linear relationship with the pH of the mobile phase. However, it is envisaged that, if the pH range was expanded further, i.e. pH 1–8, the capacity factor of these three weak electrolytes should have a sigmoidal function with the pH of the eluent [7]. As shown in eqns. 1–3 and Fig. 1a–c, the ln k' of the three compounds has a quadratic relationship with the volume fraction of the acetonitrile of the eluent. Such an expression has been reported by several workers and postulated to be caused by a dual retention mechanism [8–11].

In order to evaluate the predictive power of eqns. 1–3, four more experiments, using a 2 × 2 factorial design, were carried out to compare the observed and the

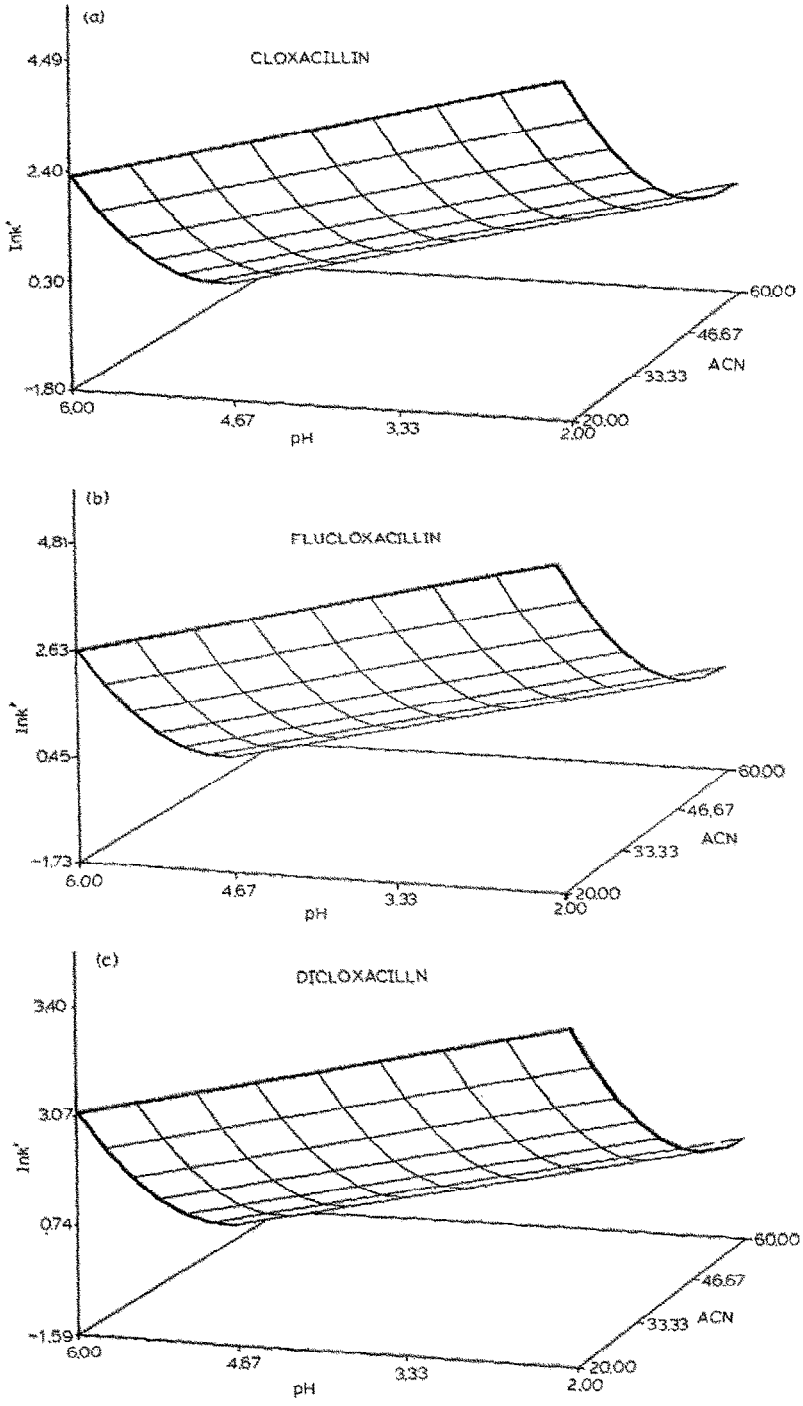

Fig. 1. Capacity factor ($\ln k'$) maps for the three isoxazotyl penicillins as a function of the pH and acetonitrile (ACN) concentration of the eluent. Chromatographic conditions as in Table I.

TABLE II

COMPARISON OF ln k' OBSERVED AND ln k' PREDICTED FOR THE THREE ISOXAZOLYL PENICILLINS

C = cloxacillin; F = flucloxacillin; D = dicloxacillin. Chromatographic conditions as in Table I.

Mobile phase		ln k'					
pH	% Acetonitrile	Observed			Predicted (mean ± S.D.)		
		C	F	D	C	F	D
3	30	2.19	2.43	2.85	2.15 ± 0.36	2.40 ± 0.37	2.82 ± 0.38
3	50	0.20	0.30	0.47	0.10 ± 0.36	0.23 ± 0.37	0.45 ± 0.38
5	30	0.74	0.94	1.02	1.06 ± 0.36	1.30 ± 0.37	1.68 ± 0.38
5	50	−0.83	−0.83	−0.53	−0.99 ± 0.36	−0.87 ± 0.37	−0.68 ± 0.38

predicted ln k' of the solutes. The results are presented in Table II. This table indicates that all the measured retention data fall within the corresponding 95% confidence interval of the predicted values and this confirms the predictive capability of the derived equations.

The derived equations (1–3) were then employed for the optimization of the retention and separation of the three solutes. Contour plots of the k' of the compounds between 4 and 20 were generated and are shown in Fig. 2. The generated experimental conditions were further reduced by using the resolution function (R_s) as the separation performance criterion. Only those conditions that could provide R_s between 1.2 and 5 for the adjacent pairs of compounds, i.e. cloxacillin and flucloxacillin, and flucloxacillin and dicloxacillin, were considered. The R_s of the adjacent solute pairs was calculated using the following equation:

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'}{k' + 1}$$

The resolution maps for cloxacillin and flucloxacillin, and flucloxacillin and dicloxacillin are shown in Fig. 3. As shown in Fig. 3a and b, mobile phases within region A will provide adequate resolution for the three solutes at the shortest analysis time. For convenience, a mobile phase with pH 2 and 40% (v/v) acetonitrile was used for the subsequent analysis of flucloxacillin in human plasma. A typical chromatogram of the three solutes obtained using this mobile phase is shown in Fig. 4.

Analysis of flucloxacillin in human plasma

Sample pretreatment. Several sample preparation methods for flucloxacillin and the internal standard, dicloxacillin, in human plasma were investigated. Injection of the samples after protein precipitation was initially studied [12]. Removal of the plasma protein and interfering substance by the addition of excess ammonium sulfate crystals was ineffective. Addition of trichloroacetic acid and

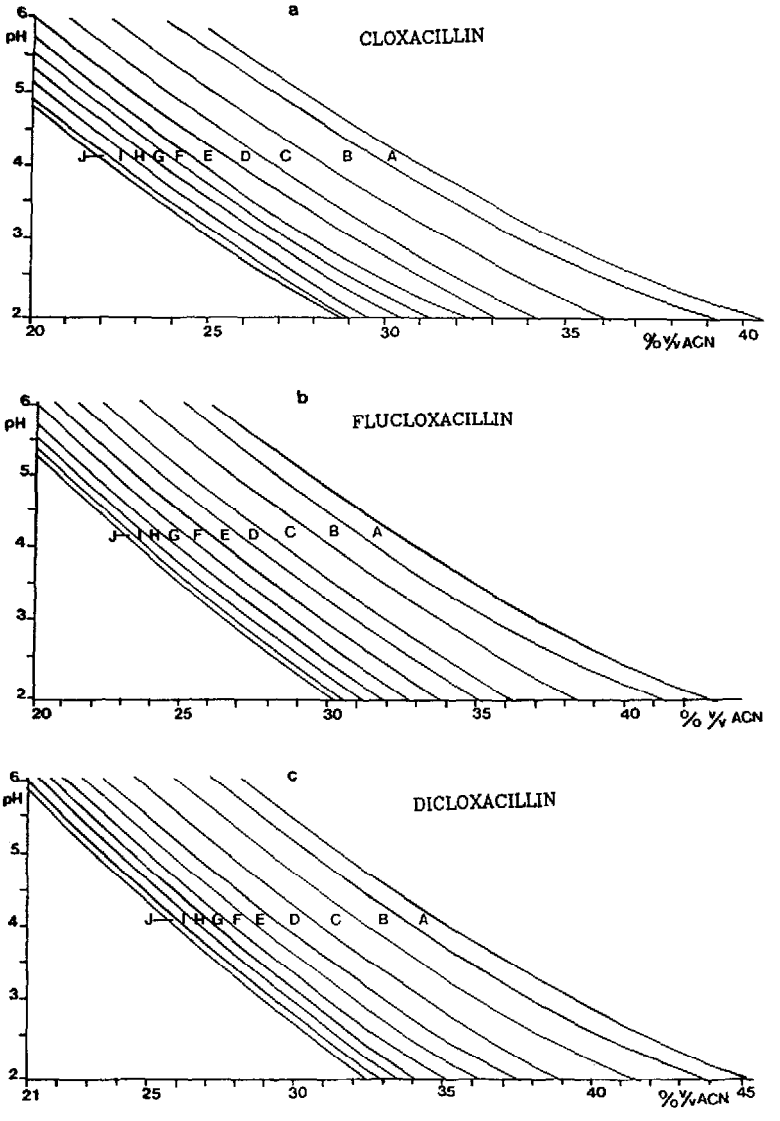

Fig. 2. Experimental conditions that will provide the k' of the three drugs between 4 and 20. The capacity factors of the solutes in the different regions are: (A) 4.0–4.9; (B) 4.9–6.6; (C) 6.6–8.4; (D) 8.4–10.2; (E) 10.2–11.9; (F) 11.9–13.7; (G) 13.7–15.4; (H) 15.4–17.2; (I) 17.2–18.9; (J) 18.9–19.9. Chromatographic conditions as in Table I.

perchloric acid [100 μl of 60% (w/v) to 1 ml of plasma] were found to precipitate the drug as well as the plasma protein. Diluting the plasma with twice the volume of acetonitrile or methanol followed by centrifugation at 8000 g for 10 min was rather effective in the removal of interfering substances. However, a delayed endogenous peak was detected at approximately 13 min (see Fig. 5). Although this unknown peak did not interfere with the analysis, it prolonged the running time of the assay. In addition, severe peak broadening of flucloxacillin and dicloxacillin was observed. This was due to the relative high concentration of organic mod-

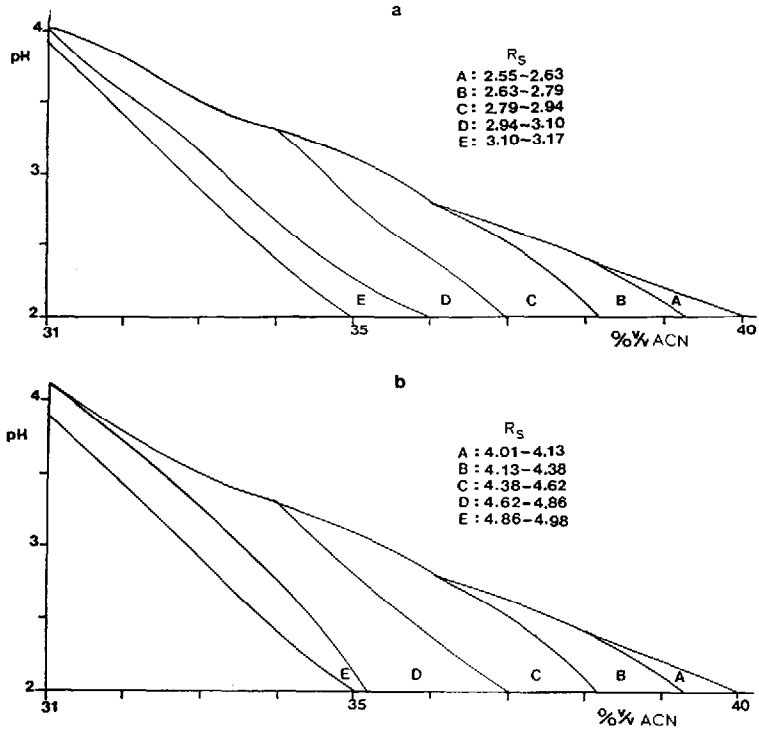

Fig. 3. Experimental conditions that will provide resolution (R_s) between 1.2 and 5 for (a) cloxacillin and flucloxacillin and (b) flucloxacillin and dicloxacillin. The capacity factors for each solute were restricted to 4 and 20. Chromatographic conditions as in Table I. ACN = acetonitrile.

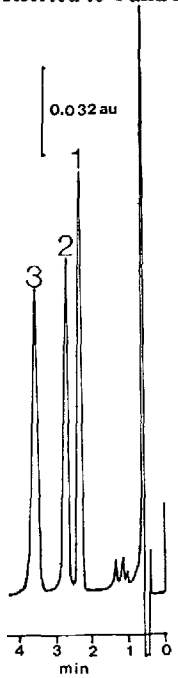

Fig. 4. Chromatographic separation of the three isoxazolyl penicillins. Peaks: 1 = cloxacillin; 2 = flucloxacillin; 3 = dicloxacillin. Concentration of each solute was 10 μg/ml and injection volume 20 μl in water. For chromatographic conditions see text.

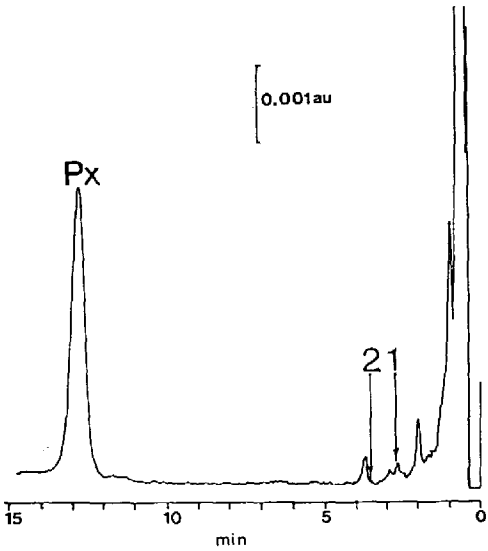

Fig. 5. Representative chromatograms of human blank plasma extract after diluting 1 ml of plasma with 2 ml of acetonitrile or methanol. Injection volume was 20 μl. Peaks: 1 = flucloxacillin; 2 = dicloxacillin; Px = unknown endogenous peak. For chromatographic conditions see text.

ifier content in the sample [13]. Extraction of drugs from acidified plasma using diethyl ether, chloroform or methylene chloride followed by evaporation and reconstitution did not result in any significant improvement of the assay or eliminate the unknown peak from the chromatogram. A solid-phase extraction technique was then adopted. Direct introduction of the plasma samples onto the Bond Elut C_{18} cartridges followed by successive washing with 2 ml of water, 2 ml of 10% (v/v) acetonitrile in 10 m*M* (pH 2) phosphate buffer and 1 ml of water and elution with 2×500 μl of 35% (v/v) acetonitrile in 10 m*M* (pH 6) buffer provided adequate recovery (>90%) and selectivities. However, the flow resistance of the cartridges increased rapidly, apparently due to the precipitation of plasma components. This problem was circumvented by pretreating the 250-μl plasma sample with 400 μl acetronitrile to precipitate the protein before its introduction to the C_{18} cartridge. This step not only simplified the extraction procedure but also prolonged the performance of the column. To ensure adequate retention of the solutes by the cartridge, the protein-precipitated sample was diluted with 700 μl of 10 m*M* (pH 2) phosphate buffer. If the Bond Elut C_{18} stationary phase has similar retention characteristics as ODS-Hypersil, the resultant eluent would produce k' of about 25 and 31 for flucloxacillin and dicloxacillin, respectively, calculated using eqns. 2 and 3. After washing the column with 1 ml of water to remove the acidic buffer, the solutes were then recovered (>90%) with 2×500 μl of 35% (v/v) acetonitrile in 10 m*M* (pH 6) phosphate buffer. Using this eluent the k' for flucloxacillin and dicloxacillin would be approximately 1. Washing the column with water before the solute elution is critical. Omission of this step will result in a drastic reduction in drug recovery, especially the internal standard. Fig. 6 shows the chromatograms of a subject blank plasma

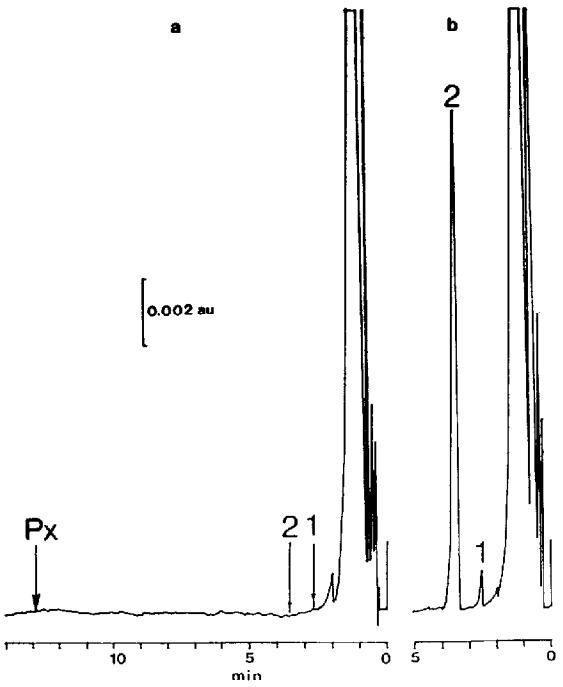

Fig. 6. Representative chromatograms of (a) human blank plasma processed by protein precipitation and solid-phase extraction and (b) plasma with 0.25 μg/ml flucloxacillin after extraction. Peaks as in Fig. 5. Injection volume was 25 μl. For chromatographic conditions see text.

sample and human plasma with known quantity of flucloxacillin and the internal standard. Fig. 6 also demonstrate that this sample pretreatment procedure increases the sensitivity of the analysis by reducing the interfering endogenous substances. In addition, the analysis time is reduced, due to the elimination of the delayed endogenous peak. Using this method, only ten cartridges were required in this study to process over 400 plasma samples and the cartridges showed no sign in decreasing in separation efficiency or increasing flow resistance.

Linearity, precision and sensitivity. The standard curve (0.2–50 μg/ml) was linear with coefficient of determination greater than 0.98. The within-day coefficient of variation based on three determinations at 0.5, 5 and 25 μg/ml was less than 7%. Taking a signal-to-noise ratio of 2 as the criterion, the detection limit of this assay for flucloxacillin was about 0.1 μg/ml.

Bioavailability study. Fig. 7 shows the mean plasma concentration of flucloxacillin sodium in ten volunteers after the oral ingestion of 500-mg flucloxacillin capsules from the two sources. Comparison of the bioavailability parameters of flucloxacillin, following the administration of the two dosage forms, using paired *t*-test indicated no differences at 5% level. For both capsules the peak plasma level, time to peak level, area under the plasma concentation–time curve and the harmonic elimination half-life [14] were approximately 22 μg/ml, 1 h, 48 μg·h/ml and 1.4 h, respectively. Therefore the two dosage forms can be considered bioequivalent.

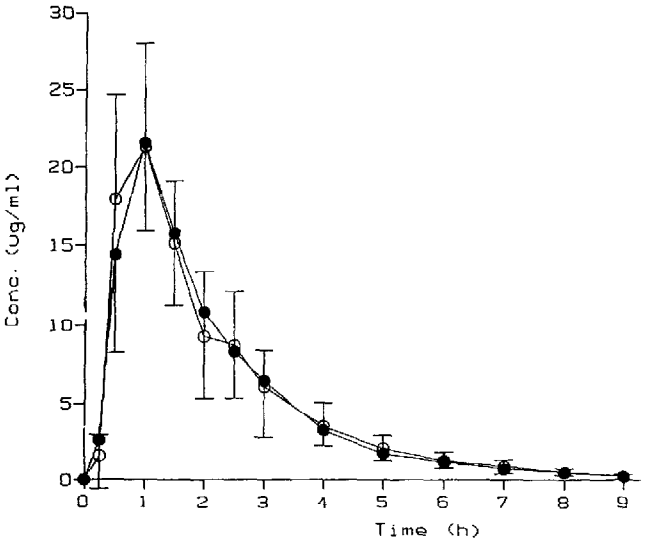

Fig. 7. Mean plasma flucloxacillin concentration resulting from the oral administration of one 500-mg Floxapen (○) and one 500-mg generic flucloxacillin capsule (●) to each of the ten subjects. The bar indicates 1 S.D. from the mean.

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