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Short communication

Rapid column liquid chromatographic analysis of flucloxacillin in plasma on a microparticulate pre-column

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Abstract

A rapid, sensitive, accurate and precise high-performance liquid chromatographic assay is described for flucloxacillin in plasma. Samples (100 μl) containing dicloxacillin (internal standard) were extracted with ethyl acetate (2 ml). The mobile phase of acetonitrile (18%, v/v) in phosphate buffer (0.01 *M*, pH 7) was pumped at 1.2 ml/min through a 40 × 3.2 mm I.D. column (3 μm particles). Detection was at 220 nm. Calibration plots were linear ($r > 0.9995$) from 0.2 to 40 mg/l. Within-day and between-day coefficients of variation were less than 9% ($n = 6$). The detection limit was 0.05 mg/l and the limit of quantitation was 0.3 mg/l. Of 24 drugs tested, only phenytoin and carbamazepine may interfere in some patients' samples.

1. Introduction

Flucloxacillin [1], a narrow-spectrum, isoxazolyl penicillin is active against penicillinase-resistant staphylococci which can complicate the treatment of infections such as osteomyelitis, septicaemia and cellulitis. In addition, antibiotic prophylaxis before and after surgical procedures frequently involves the administration of oral or intravenous flucloxacillin.

The analysis of flucloxacillin in published pharmacokinetic studies has relied mainly on a variety of microbiological methods [1–3] which are unselective and imprecise compared with more recent approaches using high-performance liquid chromatography (HPLC) [4,5]. We describe here a sensitive and precise HPLC method for the determination of flucloxacillin in plasma using ethyl acetate extraction from acidified plasma followed by reversed-phase chromatography on a 40 mm microparticulate pre-column. Compared with other HPLC methods, this simple and robust approach offers savings in sample processing and chromatographic run times, and in overall assay cost.

2. Experimental

2.1. Reagents and materials

Flucloxacillin sodium was a supplied by Alphapharm (Carole Park, Qld, Australia). Dicloxacillin sodium was purchased from Sigma (St. Louis, MO, USA). 5-Hydroxymethyl flucloxacillin was obtained from Beecham Research Lab-

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oratories (Dandenong, Vic, Australia). Orthophosphoric acid (85% w/w) was obtained from BDH Chemicals Australia (Kilsyth, Vic, Australia). Acetonitrile and ethyl acetate were HPLC grade from Mallinckrodt Australia, (Clayton, Vic, Australia). Glass-distilled water was used throughout.

To prepare standards, a master stock solution of flucloxacillin sodium was prepared in distilled water containing 400 mg/l flucloxacillin acid equivalents. A stock solution of dicloxacillin sodium containing 10 mg/l of dicloxacillin acid equivalents was similarly prepared. Flucloxacillin standards were prepared in drug-free plasma to contain 40, 20, 10, 5, 2, 1, 0.5 and 0.2 mg/l flucloxacillin. Seeded controls were prepared from a fresh master stock to contain 30, 3 and 0.3 mg/l flucloxacillin in plasma.

2.2. Instrumentation

A modular HPLC system was used comprising a Model M45 pump, a Model 712 (WISP) autosampler and a Model 484 variable wavelength detector, all supplied by Millipore (Waters Chromatography Division, Milford, MA, USA). Chromatograms were recorded on an Omniscribe chart recorder (Houston Instruments, Austin, TX, USA).

2.3. Sample preparation

Plasma (100 μl), internal standard solution (100 μl) and glacial acetic acid (25 μl) were pipetted into a 13 × 100 mm glass tube. Ethyl acetate (2 ml) was added and the contents vortex-mixed for 30 s and then centrifuged at approximately 2000 *g* (5 min). The clear supernatant was decanted into a second tube and evaporated at 70°C in a heating block under a gentle flow of nitrogen via an overhead manifold. The residue was dissolved in 250 μl of mobile phase of which 10–20 μl were injected.

2.4. Chromatography and quantitation

All HPLC analyses were performed at 22 ± 2°C. The mobile phase of acetonitrile–phosphate buffer (0.01 *M*, pH 7) (18:82, v/v) was pumped at 1.2 ml/min through a Brownlee RP18 VeloSep cartridge column (40 × 3.2 mm) containing spherical, porous, 3-μm particles (Applied Biosystems, Foster City, CA, USA). The mobile phase was filtered (0.45 μm pore size) and degassed under reduced pressure before use. Peaks were detected at 220 nm using a sensitivity range of 0.005–0.02 AUFS. The recorder was set for a 10-mV input and chart speed of 2.5 mm/min.

Calibration equations were constructed by weighted linear regression analysis [6] of the peak height ratio of flucloxacillin to dicloxacillin versus flucloxacillin concentration in plasma standards.

3. Results and discussion

This assay was developed to address deficiencies in previously published methods for the measurement of flucloxacillin in plasma. Microbiological assays demand specialised sterile environs, strict aseptic technique and overnight incubation of test cultures. They also lack specificity and precision and for these reasons alone their replacement with liquid chromatographic methods has been strongly recommended [7]. Of these HPLC methods, the description of the procedure of Thijssen [4] provided no linearity, recovery and imprecision data while the method of Hung and co-workers [5] required extensive sample cleanup using relatively expensive solid-phase extraction. Neither method gave any indication of the potential for interference from other drugs in clinical samples.

Presently, flucloxacillin and the internal standard, dicloxacillin, had retention times of 2.8 min and 4.4 min, respectively, and were eluted well clear of other peaks (Fig. 1). The 5-hydroxymethyl metabolite of flucloxacillin eluted at 1.4 min in the solvent front, but was not measured since its area under the plasma concentration–time curve was less than 4% of that of flucloxacillin [4].

Calibration plots were linear ($r > 0.999$, $n = 6$) from 0.2 to 40 mg/l (Table 1). It was necessary

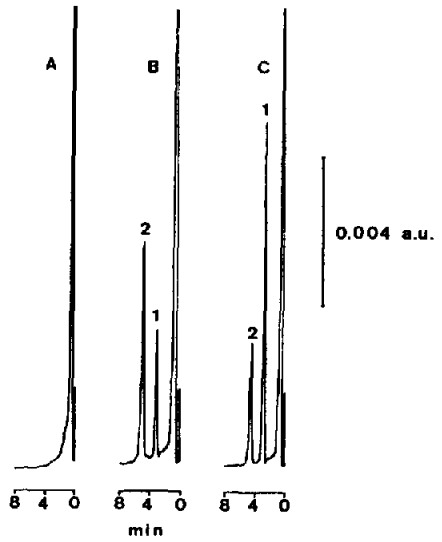

Fig. 1. Chromatograms of (A) drug-free plasma, (B) plasma standard containing 5 mg/l flucloxacillin, and (C) plasma sample containing 30 mg/l flucloxacillin drawn 30 min after a 2-g intravenous bolus of flucloxacillin sodium. The detector sensitivity is indicated by the scale bar. Peaks: 1 = flucloxacillin; 2 = dicloxacillin (internal standard).

to weight the regression analyses by a factor proportional to the reciprocal of the variance [6] to avoid grossly inaccurate estimations of flucloxacillin at low concentrations. For example, a typical standard curve of unweighted data: $y = 0.107x + 0.023$, gave deviations from target values of 0.3 mg/l and 3 mg/l of −53.3% and −10.0%, respectively. In contrast, the corresponding weighted regression: $y = 0.111x - 0.002$, gave deviations of only +3.3% and −6.7% for these seeded controls. Analysis of variance of replicate calibration data ($n = 6$) showed that the error variance term contained statistically insignificant non-linear elements. The analysis (in no fixed order) of 6 sets of seeded controls on the same day and 1 set of controls once daily for 6 days, gave acceptable within-day and between-day imprecision of less than 9% coefficient of variation (Table 2). While the limit of quantitation of 0.3 mg/l theoretically could be decreased (e.g. by increasing plasma volume and/or amount injected) the assay is presently sensitive enough to measure plasma flucloxacillin concentrations for at least 7 half-

Table 1
Weighted and unweighted regression analysis of 6 replicate calibration plots

Method	α	β	r
Unweighted[a]	0.028 ± 0.006	0.100 ± 0.004	0.9997 ± 0.0003
Weighted[b]	0.107 ± 0.006	0.003 ± 0.005	

Data are expressed as mean ± S.D.
[a] $Y_i = \alpha + \beta \cdot (X_i)$
[b] $Y_i/X_i = \beta + \alpha.(1/X_i)$ (See ref. 6)

Table 2
Imprecision and inaccuracy of flucloxacillin HPLC assay

Target flucloxacillin concentration (mg/l)	Imprecision (C.V.%; $n = 6$)		Inaccuracy[a] (%)
	Within-day	Between-day	
0.3	7.0	6.8	−6.8
3	8.8	6.8	+4.5
30	5.9	3.0	+7.7

[a] ([Target conc. − calculated conc.]/target conc.) · 100%.

lives after the peak following administration of 500-mg oral doses [5]. The limit of detection was 0.05 mg/l, equivalent to a peak height of three times the baseline noise. Recovery of flucloxacillin from plasma (0.3–30 mg/l) was 60–70% when peak heights after extraction were compared with those when flucloxacillin controls were injected in mobile phase. Extraction of flucloxacillin from plasma with ethyl acetate was preferable to the dichloromethane used in a previous method [4], since dichloromethane formed an emulsion with plasma which had to be carefully aspirated before the lower (dichloromethane) layer could be isolated. In contrast, denatured plasma from ethyl acetate extraction formed a solid, crusty residue which adhered to the bottom of the tube after centrifugation thereby allowing easy removal of the organic layer without risk of contamination from plasma residues.

The potential for interference from coadministered medication was investigated by extraction and chromatography of plasma samples supplemented with a variety of commonly monitored drugs at concentrations encountered clinically (Table 3). Of these, potential interference was noted with phenytoin (22 mg/l) and carbamazepine (16.4 mg/l) which had retention times close to that of the internal standard. This finding was confirmed by assaying samples from several epileptic patients taking these drugs. No extra peaks attributable to the respective major metabolites *p*-hydroxydiphenylhydantoin and carbamazepine-10-epoxide were observed. Dexamethasone (retention time 7.6 min) has the potential for use as an alternative internal standard since it is soluble in ethyl acetate and is detectable at 220 nm. Neither of the 2 previously published HPLC methods [4,5] screened other drugs for potential interference.

Hitherto, 40-mm VeloSep cartridges have been used as pre-columns to prevent components in biological matrices from contaminating the main analytical column, although a very recent paper described their use in the assay of cyclosporine A in whole blood during routine monitoring [8]. A major advantage of the present method is that substantial savings in solvent and column costs can be realised. The VeloSep pre-column is less than half the price of many 150–300 mm analytical columns and requires substantially less acetonitrile in the mobile phase, compared with other HPLC procedures. The method is rapid in that 60 standards, controls and samples can be

Table 3
Screening of drugs for interference in flucloxacillin HPLC assay

Drug	Concentration (mg/l)	t_R^a (min)	Drug	Concentration (mg/l)	t_R^a (min)
Acetaminophen	220	< 1	Nortriptyline	125[b]	n.d.[d]
Amikacin	23	n.d.	Methotrexate	12[c]	n.d.
Amitriptyline	130[b]	n.d.	Phenobarbital	60	1.2
Caffeine	10.2	< 1	Phenytoin	22	4.4
Carbamazepine	15	4.4	Primidone	15	< 1
Chloramphenicol	13.6	n.d.	Procainamide	11.6	n.d.
Cyclosporine	270[b]	n.d.	Quinidine	5	n.d.
Digoxin	3.8[b]	n.d.	Salicyclate	600	n.d.
Ethosuximide	85	< 1	Theophylline	24	< 1
Gentamicin	6	n.d.	Tobramycin	10.5	n.d.
Lidocaine	7.6	n.d.	Valproic Acid	83	n.d.
N-Acetylprocainamide	19	n.d.	Vancomycin	24	n.d.

[a] Retention time (t_R of flucloxacillin, 2.8 min; t_R of dicloxacillin, 4.4 min).
[b] μg/l.
[c] μmol/l.
[d] Not detected.

extracted and chromatographed in batch mode during a normal working day. Analysis of a single sample requires approximately 25 min.

In conclusion, this method would be useful in pharmacokinetic and bioavailability studies of flucloxacillin and in the analyses of samples drawn from individual patients during clinical investigations. The use of a small (100 μl) sample volume offers scope for further application in paediatric and neonatal populations.

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